Evaluation of oral streptococci in saliva of children with severe Early Childhood Caries and caries-free



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Abstract

Aim Oral streptococci were found to be associated with Early Childhood Caries. The aim of this study was to evaluate the 6 different bacteria in the streptococcus group in the saliva of children with severe early childhood caries (S-ECC) by polymerase chain reaction (PCR).

Materials and methods A total of 60 children between 3 and 6 years of age were divided into two groups: children with S-ECC (Group S-ECC; n=30) and children who were caries-free (Group CF; n=30), according to the dmft and dmfs indices. Unstimulated saliva was collected from all participants for the detection of streptococcal group bacteria, including: Streptococcus mutans, Streptococcus oralis, Streptococcus sanguinis, Streptococcus gordonii, Streptococcus salivarius, and Streptococcus sobrinus, using PCR-restriction fragment length polymorphism (RFLP) of amplified 16S rRNA gene. The data were analysed using SPSS software.

Results The prevalence of S. oralis was significantly higher in the S-ECC group compared to the CF group ($p<0.05$). However, the frequencies of S. mutans, S. sanguinis, S. gordonii, S. salivarius, and S. sobrinus were similar between the two groups ($p>0.05$). The amount of streptococci colonies was higher in the S-ECC group compared to the CF group ($p<0.05$).

Conclusion S. mutans or S. sobrinus alone may not be the only indicators for high risk of caries, but the prevalence of S. oralis in saliva may be a risk factor for increased caries activity in S-ECC.

KEYWORDS Early childhood caries, Microbiology, Polymerase Chain Reaction, Saliva, Streptococci.

Introduction

Early Childhood Caries (ECC) was reported as the most common chronic disease seen in childhood, with an increase of newly diagnosed cases of 1.8 billion per year worldwide [Fakhruddin et al., 2018; Xiao et al., 2018]. Severe ECC (S-ECC) is defined as the existence of one or more cavitated teeth, missing teeth caused by dental caries or filled smooth surfaces in primary maxillary anterior teeth, or decayed, missing, or filled score of ≥ 4 (age 3), ≥ 5 (age 4), or ≥ 6 (age 5) surfaces [Policy on Early Childhood Caries, 2016] Dental United States 2016 Oct 1942-5473 (Electronic).

Despite protective strategies to prevent dental caries, ECC has still been a significant health problem in developed and developing countries [Colak et al., 2013]. In aepidemiological studies evaluating the frequency of dental caries observed in different countries, the global increase of the prevalence of ECC has been emphasised [Bagramian et al., 2009]. The World Health Organization (WHO) has proposed that at least 80% of children aged 6 years should be caries free by 2020 [Ma et al., 2015]. However, in terms of primary dentition, Turkish children were found to be very far from this aim. Therefore, it was suggested that strategies for prevention of the disease are needed urgently [Zhou et al., 2011]. Dental caries was found to be associated with the primary cariogenic bacteria S. mutans and S. sobrinus in the mutans streptococci (MS) family [Fakhruddin et al., 2018]. However, other oral streptococcus bacteria, including S. sanguinis, S. oralis, S. gordonii, and S. salivarius [Hoshino et al., 2004], were also reported to affect cariogenicity through modulating the biofilm community and interacting with MS during the caries process [Kuramitsu and Wang, 2006]. Thus, understanding the role of oral streptococci in caries may gain importance for prevention of the disease [Martinez-Martinez et al., 2012; Meriç, 2018]. Previous research evaluated dental plaque to identify the role of streptococcal species in S-ECC [Fakhruddin et al., 2018; Xu et al., 2014]. However, it was claimed that dental plaque does not reflect the oral bacteria/MS prevalence completely [Lindquist and Emilson, 1990]. Thus, the use of saliva as an alternative to plaque was suggested because it is superior in reflecting MS colonisation for the entire dentition [Nurelhuda et al., 2010]. Because of its surface antigen proteins, MS was

found to persist in saliva [Petersen et al., 2002]. In addition, these sequencing techniques were used successfully for saliva analysis in research evaluating caries [Ge et al., 2008; Jiang et al., 2016; Ma et al., 2015].

The aim of this study was to evaluate the oral streptococci in S-ECC and caries-free (CF) children to identify the organisms involved in tooth decay for prevention of ECC in Turkish preschool children using PCR-restriction fragment length polymorphism (RFLP) of amplified 16S rRNA gene. The hypothesis of the current study was that not only S. mutans and other oral streptococci were involved with ECC.

Materials and methods

Study population

A total of 60 children between 36 and 71 months of age, without any physical or mental disability, for whom topical antibiotic or fluoride were not applied for the last month, were included in the study. Thirty children (11 girls and 19 boys) were specified as S-ECC group according to the criteria accepted by the American Academy of Pediatric Dentistry one or more decayed (noncavitated or cavitated lesions), missing (due to caries) or filled tooth surfaces in any primary tooth [Policy on Early Childhood Caries, 2016]. The control group consisted of 13 female and 17 male patients with no caries of similar age groups (no carious symptoms were observed for 5 seconds with air freshener) [Bhoopathi, 2017]. Teeth lost due to trauma and/or undergoing physiological root resorption were excluded. The ethical approval was obtained for this study from the Ethics Committee of Mustafa Kemal University (#2016-74). A detailed written informed consent was obtained from the parents of all participants.

Patient evaluation and caries index

An oral and dental health examination was performed on all participants by the same examiner (E.M.) in the Department of Pediatric Dentistry, Faculty of Dentistry, Mustafa Kemal University (Hatay, Turkey). The examination was performed on a dental chair under reflector light, using a mouth mirror and explorer, as previously described in the literature [Martinez-Martinez et al., 2012].

Firstly, teeth were isolated with rolled cotton and then gently dried with air-water spray for five seconds to make all decay lesions easily detectable. Then, the dmft and dmfs values were recorded in accordance with the criteria determined by the WHO (1997) in order to determine the pretreatment status of the children. Teeth lost because of trauma and physiological root resorption were excluded. Oral hygiene and nutritional behaviours were provided to the patients and parents in both groups. Routine treatment procedures on the teeth of the children in both groups were performed after the collection of saliva samples.

Collection of microbiological samples

All participants were placed in an upright position with their heads tilted forward. Saliva accumulated at the base of the mouth, allowing for unstimulated saliva samples to be taken. The patients were told to stay still without moving their tongue, lips, or cheeks to collect and then spit out the saliva [Jiang et al., 2016]. Saliva sampling was carried out at 9–11 AM with the dental examination. No food was permitted to consume within 1 h before saliva collection in order to avoid circadian rhythm changes. One ml of non-stimulated saliva was obtained from each child [Ghasempour et al., 2013]. The samples were sent to the culture laboratory of the Microbiology Department under cold chain for microbiological cultivation and evaluation.

Microbiological analysis of the samples

All samples were vortex-mixed followed by a sonication, and plated on mitis salivarius agar as previously described in the literature [Jiang et al., 2016]. Following a 72 h of incubation at 37 °C in an anaerobic atmosphere of 85% N_2, 10% H_2, and 5% CO_2, colony-forming units (CFU) were enumerated for the estimation of total oral streptococci on mitis salivarius agar according to the manufacturer's recommendations [Ge et al., 2008].

DNA extraction and PCR procedure

Salivary DNA was extracted and quantified as previously described in the literature [Martinez-Martinez et al., 2012]. The existence of GTF genes in the DNA was determined with the aid of species-specific GTF primers [Hoshino et al., 2004] (Table 1). PCR procedure was carried out in in 25 µl of a reaction mixture with the conditions previously used in the literature [Martinez-Martinez et al., 2012].

16S rRNA genes PCR-RFLP and 16S rRNA gene sequencing

Streptococcies were identified using restriction fragment length polymorphism analysis (RFLP) of PCR-amplified 16S ribosomal RNA genes. 16S rRNA gene sequences were amplified by PCR using primers from genomic DNA and digested PCR products with the restriction HaeIII [Sato et al., 2003]. 16S rRNA of S. mutans NCTC10449 reference strain was used as positive.

Imaging of PCR products by electrophoresis

PCR products were evaluated by electrophoresis in running them in 2% agarose gel with the aid of Tris-acetate-EDTA buffer, using a 100-bp DNA ladder marker (Gene Ruler, Thermo Scientific, Massachusetts, USA). All gels were stained with ethidium bromide (0.5µg/ml) and photographed under UV illumination (Wealtec, Dolphin-View, Nevada, USA) as previously described in the literature [Martinez-Martinez et al., 2012].

Statistical analysis

To estimate the sample size, a power analysis was performed using a programme (G*Power version 3.1.7, Franz Faul, Christian-Albrechts-University, Kiel, Germany). According to the results, a total sample size of 60 participants would provide a power of 92% (actual power, 0.9217; critical F, 1.671; noncentrality parameter, 3.098) to detect a significant difference with an effect size of 0.8 at a significance level of $\alpha = 0.05$. For the statistical analysis of the data obtained in the study, SPSS software (Version 22, SPSS Inc., Chicago, IL, USA) was used. In the evaluation of the study results, the Student's t-test, Mann-Whitney U test, Chi-square test, and Fisher's Exact test were used. Significance was considered to be $p < 0.05$.

Results

Study population

A total of 60 patients, 24 girls (39.9%) and 36 boys (60.7%), were included in the study. The ECC group consisted of 30 children, 11 girls (35.5%) and 19 boys (64.5%), with a mean age of 5.03 ± 0.7 years. The healthy control group

Primer	Target Gene	Primer Sequencing (5'-3')	Fragment Length (bp)
MKD-F	S. mutans gtfD	GGCACCACAACATTGGGAAGCTCAGTT	433
MKD-R		GGAATGGCCGCTAAGTCAACAGGAT	
MKT-F	S. sobrinus gtfT	GATGATTTGGTCTCAGGATCAATCCTC	328
MKT-R		ACTGAGCCAGTAGTAGACTTGGCAACT	
MKK-F	S. salivarius gtfK	GTGTTGCCACATCTTCACTGCCTTCGG	544
MKK-R		CGTTGATGTGCTTGAAAGGGCACCATT	
MKP-F	S. sanguinis gtfP	GGATAGTGGCTCAGGGCAGCCAGTT	313
MKP-R		GAACAGTTGCTGGACTTGCTTGTC	
MKR-F	S. oralis gtfR	TCCCGGTCAGCAAACTCCAGCC	374
MKR-R		GCAACCTTTGGATTTGCAAC	
MKG-F	S. gordonii gtfG	CTATGCGGATGATGCTAATCAAGTG	440
MKG-R		GGAGTCGCTATAATCTTGTCAGAAA	

TABLE 1 Primary sequences used in PCR for streptococci.

Group	Gender	Age (Average)	dmft (Average)	dmfs (Average)
ECC (n=30)	11 Girls (35.5%)	5.03 ± 0.7	7.77 ± 3.6	23.77±16.8
	19 boys (64.5%)			
CF (n=30)	13 girls (43.3%)	4.98 ± 0.7	-	-
	17 boys (56.7%)			

CF: Caries-Free ECC: Early Childhood Caries

TABLE 2 Demographic values of study participants.

included 30 children, 13 girls (43.3%) and 17 boys (56.7%), with a mean age of 4.9 ± 0.7 years. Dmft and dmfs values of the children in the ECC group were found on average to be 7.77 ± 3.6 and 23.77 ± 16.8, respectively (Table 2).

Bacterial flora

S. mutans was observed in all children in both the ECC and control groups ($p > 0.05$) (Fig. 2). The prevalence of S. sobrinus in the ECC group (20%) was higher than in the CF group (6.7%). This did not, however, reach statistical significance ($p > 0.05$). The prevalence of S. salivarius in the ECC group (50%) was not statistically different compared to the CF group (36.7%) ($p > 0.05$). The prevalence of S. sanguinis in the ECC group (60%) did not reach statistical significance compared to group CF (40%) ($p > 0.05$). The prevalence of S. oralis in the ECC group (56.7%) was significantly higher compared to group CF (26.7%) ($p < 0.05$). There was no statistically significant difference between the prevalence of S. gordonii in the ECC group (36.7%) compared to group CF (26.3%) ($p > 0.05$) (Fig. 1). The numbers of streptococci colonies were found to be higher in group ECC (9.64 ± 1.17 x 105) than in group CF (3.69 ± 3.36 x 105) (Fig. 2).

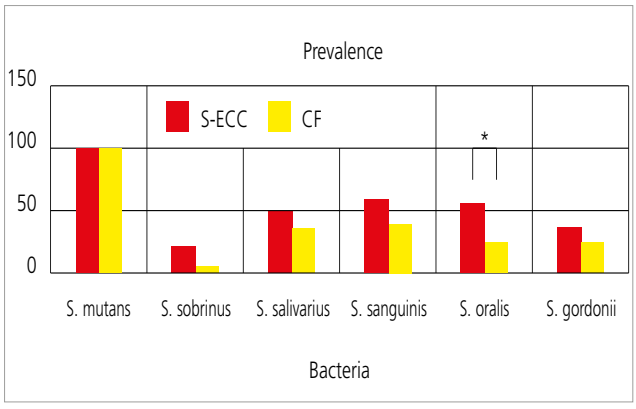


FIG. 1 The prevalence of bacteria between groups. The only significant differences in the prevalence of streptococci were detected for S. oralis between groups * $p < 0.05$.

A positive correlation was detected for the prevalence of S. sobrinus and S. mutans (100%). In addition, S. sobrinus was not isolated alone from any of the participants. No other statistically significant correlation existed between S. sobrinus and other bacteria in both groups ($p > 0.05$) (Table 3).

Discussion

S-ECC is an increasing global public health problem that requires complex treatment procedures and causes high costs [Xiao et al., 2018]. Prevention of S-ECC was suggested to correlate positively with increased understanding of its etiopathogenesis involving cariogenic bacteria [Xiao et al., 2018]. MS was reported to be involved with this caries process. In particular, S. mutans and S. sobrinus were suggested to play a major role in the cariogenic process [Ajdic et al., 2002]. However, some research found that S. mutans may not be detected in all ECC patients and may exist in CF children. In addition, other streptococci may be involved with this process [Jiang et al., 2016]. Therefore, understanding the role of other oral streptococci in S-ECC, as well as their interaction with MS, may be significant for decreasing caries risk. In this study, the numbers of streptococci colonies were found to be higher in group ECC than in group CF (Fig. 2). It is consistent with previous studies reporting higher rates of MS in children with ECC [Ghasempour et al., 2013].

With the development of molecular-based PCR methods, specific bacteria were reported to be detected more precisely than conventional culture techniques. These novel methods were also used for ECC risk assessment in children [Saraithong et al., 2015]. Thus, in the current study, the saliva of children with ECC or CF was analysed for well-known bacteria in the streptococcus family using PCR analysis. Between the evaluated streptococci, only the prevalence of S. oralis was significantly higher in group ECC compared to group CF ($p < 0.05$). However, the prevalence of S. mutans, S. salivarius, S. sanguinis, S. gordonii, and S. sobrinus were similar between groups ($p > 0.05$). Our results confirmed the notion that MS alone may not be the only indicator for caries risk. However, S. oralis may be a risk factor for high caries risk in Turkish children with S-ECC. In addition, traditional culture methods used in the current study confirmed the literature [Ge et al., 2008] that the amount of streptococci colonies was

significantly higher in group S-ECC compared to group CF ($p < 0.05$).

Previously, the aetiology of caries was not fully identified due to limited knowledge of the oral microbiome [Corby et al., 2005]. The previously used conventional cloning and sequencing techniques were found to detect only the predominant bacteria within a complex habitat [Ling et al., 2010]. However, with the development of sequencing techniques, many different microbial species in complex biological samples, including dental plaque, were identified easily [Buermans and den Dunnen, 2014]. Therefore, with the aid of these methods, knowledge concerning caries development through bacterial interactions may be understood in detail. The 16S rRNA sequence comparisons can be used to identify oral mutans streptococci; however, the identification by sequencing is sometimes difficult in large-scale studies and for small laboratories. Therefore, 16S rRNA genes PCR-RFLP, using HaeIII, could be an alternative method for the identification of mutans streptococci, and may be applicable for large-scale studies on the cariogenicity of mutans streptococci [Sato et al., 2003]. Also, it was concluded that the 16S rRNA gene offered greatest sensitivity for MS detection. Consequently, it is widely used to identify MS and many other bacterial pathogens.[Spradbery, 2010].

Studies evaluating the prevalence of S. mutans in the saliva of caries-active (CA) and CF children reported controversial results. Some of them [Luo et al., 2012; Shimada et al., 2015] stated that they did not detect S. mutans in CF children but others [Ge et al., 2008; Jiang et al., 2016; Ma et al., 2015] reported that they did isolate S. mutans in the saliva of both CA and CF children. Our results showed that S. mutans was isolated in all of the children in both the ECC group (100%) and the CF group (100%). This confirmed research that also detected S. mutans in CF children [Ge et al., 2008; Jiang et al., 2016; Ma et al., 2015]. S. sobrinus, also a member of MS, was found to be involved with S-ECC [Palmer et al., 2010] and was isolated in the saliva of children with caries. A synergistic effect was suggested between S. mutans and S. sobrinus on the exacerbation of ECC [Choi et al., 2009]. Furthermore, S. sobrinus was suggested to have more cariogenic potential than S. mutans [de Soet et al., 2000]. In our study, although all ECC patients were found to be infected with S mutans, S. sobrinus was isolated in only 6 of 30 children in group ECC and in 2 CF children. The low prevalence of S. sobrinus in our study was confirmed by Zhou et al. [2011]. They reported that the prevalence of S. sobrinus may be lower in the saliva of children aged 3–4 years but may increase with age. Similar to Zhou et al. our study population also consisted of children 2–6 years of age. Thus, the prevalence of S. sobrinus may increase with age. Qin et al. [Qin et al., 2013]detected a higher prevalence of S. sobrinus in the saliva of children with S-ECC (18.39%) compared to CF children aged 3–5 years (3.30%). This difference may be associated with the differing ethnic populations between our study and the study by Qin et al. Colombo et al. [2017] found a significantly higher prevalence in the saliva of children for S. sobrinus with S-ECC compared to CF children aged 3-6 years ($p < 0.05$). In their study, the prevalence of S. mutans was also reported to be significantly higher in the saliva of ECC children compared to CF controls ($p < 0.05$). The difference between Colombo et al. [2017] and our study may be involved with the different populations or oral environmental status, as we did not find a difference for S. mutans prevalence between groups. This may be responsible for the controversial results. Ge et al. [2008] found a significant prevalence for S. mutans but similar prevalence for S. sobrinus in the saliva of S-ECC children compared to CF controls aged 2-9 years. They explained this result by their wide age range and small sample size of 22 children. Ghasempour et al. [2013] also detected a significantly higher prevalence for S. mutans , but no difference for S. sobrinus, in the saliva of S-ECC children compared to CF controls. Loyola-Rodriguez et al. [2008] found a significantly higher prevalence both for S. mutans and S. sobrinus in the saliva of CA children compared to CF controls. In addition, no correlation was found between S. sobrinus and other bacteria in our groups. The controversial findings may be explained by our nutritional habits and the population of other studies that may affect the oral flora. S. oralis was suggested to be involved with ECC in children, and the prevalence of S. oralis was found to be higher in the dental plaque of children with ECC compared to CF children [Becker et al., 2002]. Similar to this finding, in our study, the prevalence of S. oralis in the saliva of the ECC group (56.7%) was significantly higher compared to the CF group (26.7%) ($p < 0.05$). However, Ma et al. [2015] evaluated the saliva of children with S-ECC or CF between 3 and 4 years of age and reported that S. oralis was isolated in all

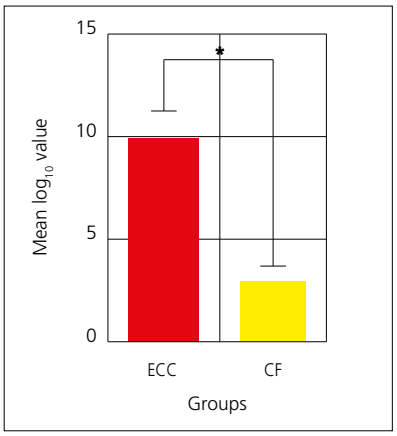


FIG. 2 Amount of streptococci colonies between groups Amount of MS colonies were significantly higher in the S-ECC group compared to the CF group. * $p < 0.05$ Data are shown as mean and standard deviation.

		Total n (%)	ECC n (%)	CF n (%)	p
Sob+sanguinis	Positive Negative	4 (%50) 4 (%50)	3 (%50) 3 (%50)	1 (%50) 1 (%50)	0.786
Sob+mutans	Positive Negative	8 (%100) 0 (%0)	6 (%100) 0 (%0)	2 (%100) 0 (%0)	-
Sob+salivarius	Positive Negative	7 (%87.5) 1 (%12.5)	5 (%83.3) 1 (%16.7)	2 (%100) 0 (%0)	0.750
Sob+oralis	Positive Negative	5 (%62.5) 3 (37.5)	4 (%66.6) 2 (%33.3)	1 (%50) 1 (%50)	0.643
Sob+gordoni	Positive Negative	3 (%60) 2 (%40)	3 (%50) 3 (%50)	0 (%0) 2 (%100)	0.357

TABLE 3 Correlation of *S. sobrinus* with other bacteria.

children in the CF group (100%). It was not, however, detected in all participants of the ECC group. The different results may be explained by the different dmfs scores of the ECC group between our study (23.77 ± 16.8) and Ma et al. (11.1 ± 5.6). Shimada et al. [2015] also reported no difference for S. oralis prevalence in the saliva of ECC children (3.6%) compared to CF children (5.6%). Although the dmft scores of our study (7.77 ± 3.6) and Ma et al. (7.0 ± 30.7) were similar, this difference for S. oralis prevalence may originate from the nutritional habits of the two different populations, namely Turkish and Japanese. Thus, it may be suggested that S. oralis was involved with S-ECC in Turkish children. S. gordonii was reported to have the capacity to inhibit S. mutans [Kreth et al., 2009]. Previously, Xiao et al. [2018] evaluated the prevalence of S. gordonii in the unstimulated saliva of 39 children with S-ECC or CF aged 3–6 years and reported no difference between the ECC and CF groups ($p > 0.05$). Similar to their findings [Kreth et al., 2009], we also did not find any difference for the prevalence of S. gordonii in the saliva of group ECC (36.7%) compared to group CF (23.3%) ($p > 0.05$). S. sanguinis was suggested to be involved with oral health-related conditions [Becker et al., 2002; Corby et al., 2005] and it had an inverse relationship with S. mutans for ECC [Kreth et al., 2009]. In addition, earlier colonisation of S. sanguinis compared to S. mutans in children was reported to cause a delay in infection with S. mutans, thus causing a delay in detecting caries [Caufield et al., 2000]. Jiang et al. [2016] and Ge et al. [2008] reported that the prevalence of S. sanguinis was similar in the saliva of CA children compared to CF children. Also, the prevalence of S. mutans was significantly higher in the CA group compared to the CF group. Similar to previous studies [Ge et al., 2008; Jiang et al., 2016; Ma et al., 2015], in our study the prevalence of S sanguinis in the ECC group (63.3%) was not statistically significantly different compared to the CF group (43.3%) in saliva ($p > 0.05$). However, contrary to their findings, the prevalence of S. mutans was similar between the groups in our study. S. salivarius was suggested to be involved with CF children and found to have a low cariogenicity [Corby et al., 2005]. However, its interactions were stated to be significant for the establishment of pathogenic plaque [Martinez-Martinez et al., 2012]. Ma et al. [2015] evaluated the saliva of children with S-ECC or CF aged 3–4 years and reported that S. salivarius was isolated in all children in the CF group (100%). It was not, however, detected in all participants of the ECC group. Martinez-Martinez et al. [2012], Shimada et al. [2015], and Xiao et al. [2018] did not find any differences for S. salivarius prevalence in the saliva of S-ECC children compared to CF children ($p > 0.05$). Similar to the literature, [Martinez-Martinez et al., 2012; Shimada et al., 2015; Xiao et al., 2018] in our study, the prevalence of S. salivarius was not statistically significant in the saliva of the ECC group (50%) compared to the CF group (36.7%) ($p > 0.05$).

Conclusion

Our study confirmed that no single bacteria, such as S. mutans, might be responsible for ECC. However, S. oralis may be an indicator of S-ECC in the saliva of Turkish children.

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