



Correlation of Streptococcus Mutans by 16SrRNA Gene Sequencing in Children with Different Caries Experience- A PCR Based Study

**Dhanalakshmi Ravikumar^{1*}, Pratibha Ramani², R. Gayathri³
and Selvaraj Jayaraman³**

¹Department of Pediatric Dentistry, Saveetha Dental College, Saveetha University, Chennai, India.

²Department of Oral and Maxillofacial Pathology, Saveetha Dental College, Saveetha University, Chennai, India.

³Department of Biochemistry, Saveetha Dental College, Saveetha University, Chennai, India.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2021/v33i41B32341

Editor(s):

(1) Dr. Ana Cláudia Coelho, University of Trás-os-Montes and Alto Douro, Portugal.

Reviewers:

(1) Zhijun Liao, Fujian Medical University, China.

(2) Sivakumar Nuvvula, Narayana Dental College and Hospital, India.

Complete Peer review History: <https://www.sdiarticle4.com/review-history/72581>

Original Research Article

**Received 08 June 2021
Accepted 13 August 2021
Published 20 August 2021**

ABSTRACT

Background and Objectives: Early Childhood Caries is considered as a common chronic disease in children. Since some research had concluded that children with higher levels of *Streptococcus mutans* are associated with a higher incidence of decayed, missing, and filled teeth, the present study was undertaken to analyze the salivary *Streptococcus mutans* of children with different caries status using species specific 16S rRNA gene sequencing technology to evaluate the quantity of *Streptococcus mutans* with respect to different caries status.

Materials and Methods: Children between 3-6 years were selected and divided into 3 groups, Group I- Caries free, Group II-Early Childhood caries and Group III- Severe Early childhood caries. The caries status was assessed using dmfs and the severity of caries was assessed using pufa index. Salivary samples were collected to isolate DNA and Real time PCR was done with 16SrRNA primer specific to *Streptococcus mutans* to estimate the quantity of *Streptococcus mutans* in children with different caries status.

*Corresponding author: E-mail: dhana9677@gmail.com;

Results: There was a significant difference in the mean CT values among the study groups ($p < 0.001$). Post hoc Tukey test revealed that, Group I had a significantly higher mean CT values than that of Groups II and III. Pearson's correlation analysis was carried out to correlate dmfs value with CT values and to correlate the pufa value with the fold change. There was a negative correlation between dmfs score and CT values and positive correlation between pufa score and fold change.

Conclusion: There was a higher expression of 16SrRNA gene in Severe Early childhood caries group, followed by Early childhood caries and caries free group, indicating a high level of *Streptococcus mutans* in Severe Early childhood caries group and there was a negative correlation between dmfs score and CT values and a positive correlation between pufa score and fold change.

Keywords: Early childhood caries; 16srRNA gene; *Streptococcus mutans*.

1. INTRODUCTION

Early Childhood Caries (ECC) is considered as a common chronic disease in children and it appears to be severe in young children due to the reduced level of immunity in these age groups [1]. The term "ECC" was suggested in 1994 at a workshop sponsored by the Centers for Disease Control and Prevention, to pay more attention towards the multifactorial aetiology of ECC namely, Socioeconomic, behavioural factors, rather than considering the sole inappropriate feeding practices. [2] The most common etiological factor of ECC is the interaction of *Streptococcus mutans* (*S.mutans*), and the cariogenic contents in the tooth Enamel. ECC is rapidly progressive and irreversible, leads to pulpal pain and infection. This can affect the chewing ability of children and nutritional status. Hence, Early diagnosis and appropriate measures are of paramount importance to prevent the negative health impacts [3-6].

While the key factors involved in the etiology of ECC are well known, *S.mutans*, colonizing the oral cavity is considered as one of the major etiological agents of dental caries [7-10]. *S.mutans* being an acidogenic and aciduric bacteria, produce acid by interacting with fermentable carbohydrates namely sucrose, fructose, glucose and cause damage to the tooth by dissolving the tooth enamel [11,12]. *S.mutans* colonize the tooth surface by variety of mechanism and it has the ability to form persisters, where it enters a state of metabolic dormancy and they neither grow nor die in the presence of high doses of bactericidal antibiotics but still able to survive [13-18]. Henceforth, *S.mutans* still remains as an enigma and preventive approaches should be designed appropriately by understanding its diversified action.

S.mutans can be identified from the oral cavity by various techniques such as, cultivation using

selective medium such as mitis salivarius bacitracin (MSB) and trypticase yeast-extract cystine sucrose bacitracin (TYCSB).[19] Apart from cultivation, modern molecular biological techniques such as Polymerase Chain Reaction (PCR) Restriction Fragment Length Polymorphism (RFLP), Denaturing Gradient Gel Electrophoresis (DGGE), Multilocus enzyme electrophoresis (MLEE), Ribotyping are most commonly used to study the *S.mutans* at genomic level [20]. These techniques of DNA sequencing and analysis are more convenient, providing more advanced and reliable results than cultivation technique.

Several studies have constantly proved that *Mutans Streptococci* (MS), primarily *S.mutans* is the prime cariogenic microorganisms associated with ECC [21-23]. Children who were colonized with MS or presented with consistently higher levels of MS may experience significantly more chance of development of ECC [24,25]. Since, *S.mutans* plays a significant cariogenic role in initiation of dental caries, numerous methods have been developed to identify and measure the colonization of *S.mutans*.

Lately, advances in 16S rRNA gene analysis have made it possible to extensively explore the microbial community and to understand its potential importance in causing the dental caries. Few studies had been carried out on PCR analysis of 16S rRNA gene together with the species-specific probes to the 16S rRNA gene to evaluate the diversity of the microbiota in children with ECC [26-29]. These techniques provide a brief insight on wide diversity of species than the cultivation methods. Since, Epidemiologic surveys have concluded that children with higher levels of *S.mutans* are associated with a higher incidence of decayed, missing, and filled surfaces (dmfs) [2,6], the present study was undertaken to analyze the salivary *S.mutans* of children with different caries

status using species specific 16S rRNA gene sequencing technology to evaluate the quantity of *S.mutans* with respect to different caries status. Secondly, to compare the possible relationship between the dmfs and pufa score and the expression of 16S rRNA gene of *S.mutans*. The study hypothesized that the expression of 16S rRNA gene varies between children with different caries status.

2. MATERIALS AND METHODS

2.1 Study Population

A total of 30 children, aged between 3-6 years were randomly selected, who reported Saveetha Dental College and Hospitals. The children were recruited based on the dmfs score. The children were divided 3 groups; Group I – Caries free (dmfs=0), Group II- ECC (dmfs=1-3) and Group III- Severe Early Childhood caries (S ECC) (dmfs>3) with 10 children in each group based on AAPD guidelines [30].

2.2 Inclusion Criteria

Children of both genders, between 3-6 years of age
Parents who provided consent by signing an informed consent
Children with no growth abnormalities

2.3 Exclusion Criteria

Uncooperative children who do not allow the examination and/or collection of saliva
Children with systemic diseases and/or pharmacological treatment

2.4 Oral Health Questionnaire

Following the parents/care givers consent, a questionnaire consisting of demographic questions such as oral hygiene practices, snacking history, nutritional habits, history of systemic illness and consumption of drugs and vitamin supplements was given to the parents.

2.5 Early Childhood Caries Diagnosis

The clinical examinations for diagnosis of ECC were performed by two calibrated paediatric dentists. Initially, theoretical discussions were done using clinical images to provide instructions on the diagnostic criteria to the Pediatric dentist. Subsequently, for the training, 10 children were examined by two pediatric dentist separately and

the Kappa coefficient was calculated. The kappa coefficient value was found to be 0.8, indicating a good agreement between the examiners. The children were seated in a calm room with adequate light and ventilation. The two trained pediatric dentists (DR and MR) examined the tooth after each tooth was cleaned and dried with sterile gauze. Disposable gloves and masks, a mouth mirror, a ball-ended dental probe and dental chair light were used to diagnose ECC. Early childhood caries was classified according to the American Academy of Pediatric Dentistry definition [30]. The caries status was recorded based on criteria of the World Health Organization Oral Health Survey Methods for Field Studies [31]. The oral health status of the children was measured using the decay–missing–filled tooth surface (dmfs) index for deciduous dentitions. as well as pulp involvement, ulceration, fistula, abscess score (pufa) index was used to measure the extent of the caries lesion [32]. For anterior teeth, 4 surfaces were examined and recorded, namely labial, Lingual/palatal, mesial and distal. For posterior teeth, 5 surfaces were examined and recorded, such as, labial, palatal or lingual, mesial, distal and occlusal. The dental examination was completed within 10 min and an assistant recorded the clinical findings.

2.6 The pufa Index Scoring System are as Follows

p/P: Pulpal involvement is considered when the opening of the pulp chamber is visible or when the coronal structures have been destroyed by the carious process and only roots and root fragments are left. No probing was performed to diagnose pulpal involvement.

u/U: Ulceration due to trauma from sharp pieces of a tooth is recorded when sharp edges of a dislocated tooth with pulpal involvement or root fragments have caused traumatic ulceration of the surrounding soft tissues, e.g., tongue or buccal mucosa.

f/F: Fistula is recorded when a pus-containing swelling related to a tooth with pulpal involvement is present.

a/A: Abscess is considered when a pus-containing swelling related to a tooth with pulpal involvement is present.

2.7 Saliva Collection

All the children were instructed to refrain from eating for two hours before sample collection.

Children were first asked to rinse their mouth with drinking water and then brush their teeth using a sterile toothbrush for 2 min. An aseptic environment was maintained throughout the sample collection. The children were asked to keep their head at 45° flexion and the saliva was allowed to drip into the tube and allowed till sufficient for analysis without measuring the froth the quantity [33]. To avoid circadian variation, the salivary samples were collected between 10.00 am -11.00 am.

2.8 DNA extraction and PCR Procedure

A QIAamp DNA Mini kit (QIAGEN Inc., USA, 9300 Germantown Road, Germantown, MD 20874) was used to extract Genomic DNA. The DNA extraction procedure was carried out according to the manufacturer's instructions of the kit. The concentration and purity of DNA solution was evaluated under UV-spectrophotometer. The DNA solution of all salivary samples was examined by 2% agarose gel electrophoresis. The extracted DNA samples were stored at - 20 °C until further processing. The 16S rRNA gene was amplified using primer specific to *S.mutans*. Smut 3368-FW 5'-GCC TAC AGC TCA GAG ATG CTAT TCT-3', Smut 3368-RW 5'-GCC ATA CACCA CTC ATGA ATTGA-3'. Quantitative RT-PCR (q PCR) was performed with the Stratagene MX3000P (Agilent technologies, 5301 Stevens Creek Blvd. Santa Clara, CA 95051). The double standard DNA-binding dye SYBR Green I (KAPA SYBR FAST qPCR Kit) and the primer specific to *S.mutans* were used. The PCR amplification product was examined using 2% agarose gel electrophoresis and 100 bp molecular marker DNA. In addition, Melt curve analysis was done to determine the specificity of the qRT-PCR, whether the PCR assays have produced a single specific amplicons. Comparative cycle threshold units (CT) method was used to calculate the amount of 16SrRNA gene. Each set of PCR analyses included a negative control (water blank). The conditions for real-time PCR were as follows: holding stage at 95°C for 10 s followed by 40 cycles of shuttle heating at 95°C for 15 s and at

60°C for 1 min. The melt curve stage was at 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. 16S rRNA was used as an endogenous control (SYBR® Green assay reagents). Relative quantification (RQ) for *Streptococcus mutans* was based on CT (the number of PCR cycles necessary to obtain the threshold signal of fluorescence) values. The quantity of *S.mutans* was expressed in CT. CT value is inversely proportional to *S.mutans* counts. Comparative C_T method used to present the gene expression of different group in fold change [34].

2.9 Statistical Analysis

All data was entered and analysed by using the SPSS 20.0 software. One way ANOVA was done to determine the difference in CT values of 16SrRNA gene between the groups. Post-hoc Tukey test was done to determine the intergroup significance. A p value of < 0.05 was considered as statistically "significant". Pearson's correlation analysis was carried out to correlate dmfs value with CT values and to correlate the pufa value with the fold change

3. RESULTS

Analysis using chi-square test indicated no statistically significant association between age and severity of decay ($p > 0.05$). The mean and standard deviation value for CT values of 16SrRNA gene is depicted in Table 1. In Group I (caries free) the mean CT value was found to be 25.33±1.93, Group II (ECC), the mean CT values were 19.93± 0.88 and Group III (S ECC), it was found to be 19.41 ±/3.02 respectively. There was a significant difference in the mean CT values among the study groups ($P < 0.001$). Post hoc Tukey test revealed that, Group I had a significantly higher mean CT values than that of Groups II and III, indicating lower levels of *S.mutans* in Group I as compared to Group II and Group III. However, no significant difference was seen between the mean CT values of Group II and Group III. (Table 2)

Table 1. Comparison of CT values of 16SrRNA between the groups

Groups	N	Mean	SD	p value
Group I (Caries-free)	10	25.33	1.93	
Group B (ECC)	10	19.93	0.88	
Group C (Severe ECC)	10	19.41	3.02	0.001**

p value was evaluated using ANOVA. p value < 0.001 – Highly significant

Fig. 1 and Fig. 2 shows the correlation of CT values of 16SrRNA gene with dmfs score of children with ECC and S ECC. There was a negative correlation between the dmfs and CT values, i.e, when the dmfs score increases there is a decrease in the CT values of 16SrRNA gene.

Figs. 3 and 4 depicts the correlation of pufa score and the fold change of *S.mutans* in children with ECC and S ECC. There was a positive correlation between the pufa score and fold change, confirming that when there is an increase in the pufa score, there is an increase the level of *S.mutans* as well.

Fig. 5 depicts the melt curve analysis of 16SrRNA gene. An amplicon from 16SrRNA gene reveals a single peak following melt curve.

4. DISCUSSION

S.mutans, the acid producing bacteria have been considered as a main pathogenic bacterium of ECC. The acid production and acid tolerance role of *S.mutans* makes it a potential cariogenic bacteria [35]. This does not mean that, the other oral streptococcus bacteria of MS family are not important. As the initial stage of caries

Table 2. Intergroup comparison of CT values of the 16SrRNA gene

Groups	Group I (Caries-free)	Group B (ECC)	Group C (Severe ECC)
Group I (Caries-free)	-	<0.001**	<0.001**
Group B (ECC)	<0.001**	-	0.850
Group C (Severe ECC)	<0.001**	0.850	-

p value < 0.001 – Highly significant – Post- hoc Tukey test

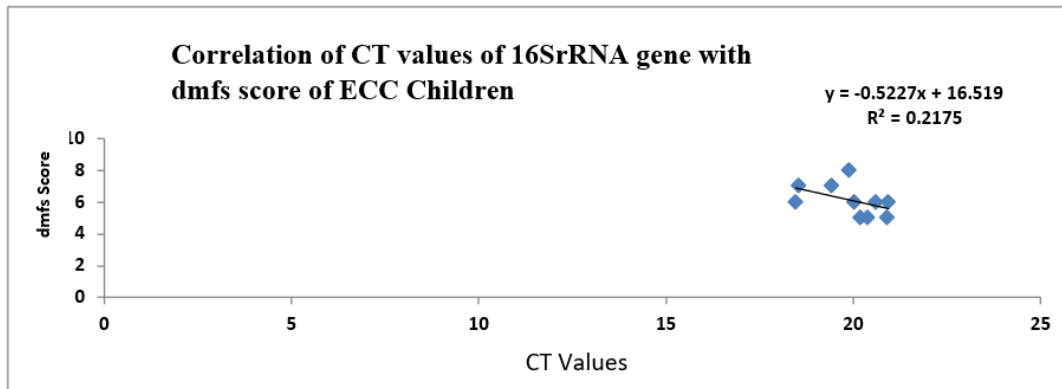


Fig. 1. Correlation of CT values of 16SrRNA gene with dmfs score of ECC children

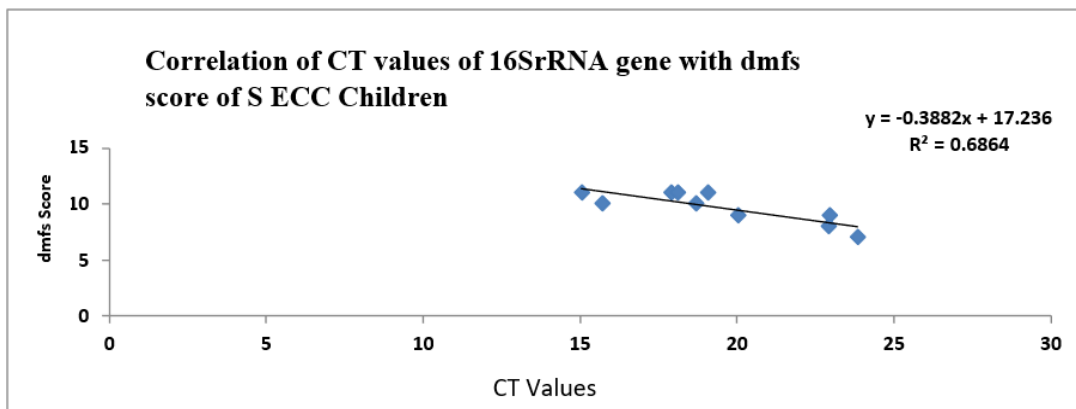


Fig. 2. Correlation of CT values of 16SrRNA gene with dmfs score of S ECC children

formation, i.e the demineralization period is dominated by *S.mutans*, most of the preventive measures should be focussed towards *S.mutans* to prevent the initiation of ECC. As the decay progresses, the local niche of bacteria changes and other oral streptococci can become a dominant microflora than *S.mutans*. Key stone pathogen theory, put forward by Hajishengallis G et al, states that, the bacteria that play a significant role in the community but appears to be scarce are considered to be the "keystone" members in the microbial ecology. Hence, low abundance of *S.mutans*, still makes it as a Key stone member [36].

Saliva plays an immense role in the prevention of dental caries and protection of oral mucosa and therefore considered as an important diagnostic tool in caries research. Lindquist and Emilson et al claimed that dental plaque does not completely reflect the MS prevalence [37].

Studies had stated that, DNA sequencing techniques for dental caries were successfully done in saliva [38-40]. In addition, Nurelhuda et al inferred that, saliva is superior in reflecting the MS colonization than dental plaque [41]. Hence, the present study utilized salivary samples to evaluate the 16SrRNA gene expression of *S.mutans*.

In the present study, caries status was assessed using dmfs and the severity of caries was assessed using pufa index. The pufa index assess the pulpo periapical extension of untreated dental caries. The upper case PUFA is used to score the permanent dentition and the lower case is used to score the primary dentition. It was developed by Monse et al [32]. The pufa index is score the presence of visible pulp (p), ulceration of the oral mucosa (u), fistula (f) or an abscess (a).

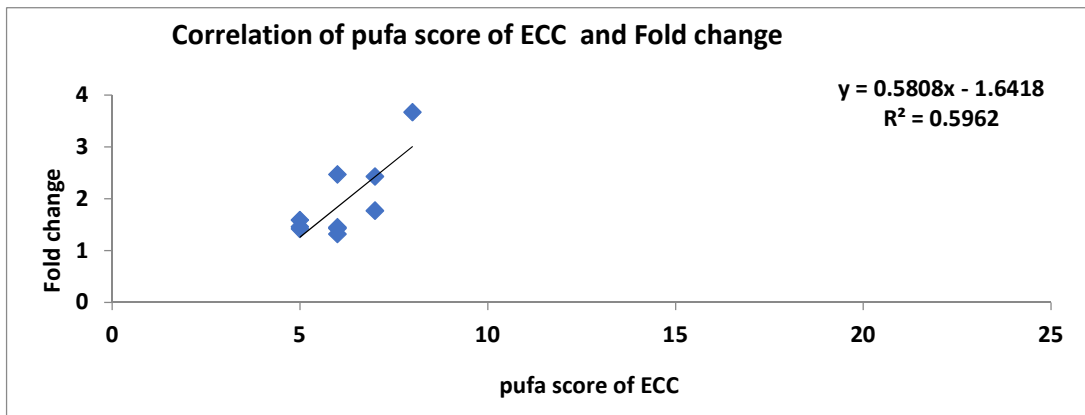


Fig. 3. Correlation of pufa score with the fold change in children with ECC

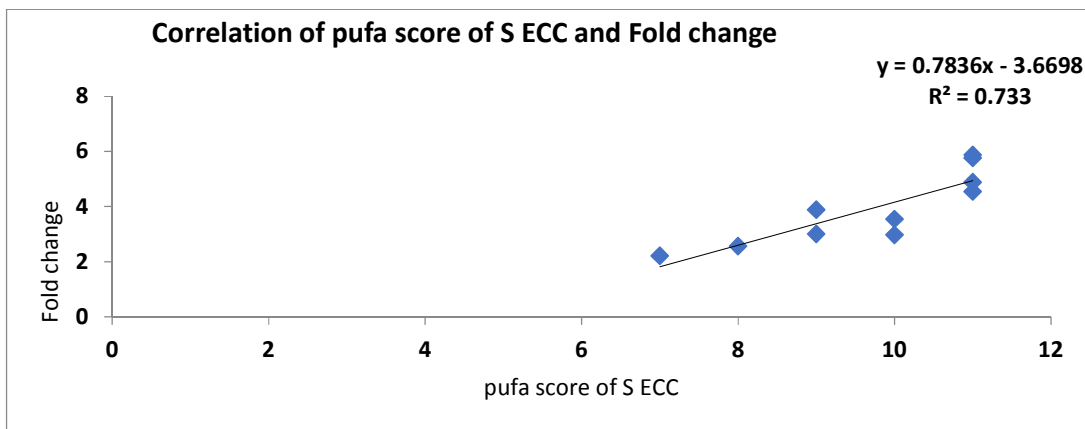


Fig. 4. Correlation of pufa score with the fold change in children with S ECC

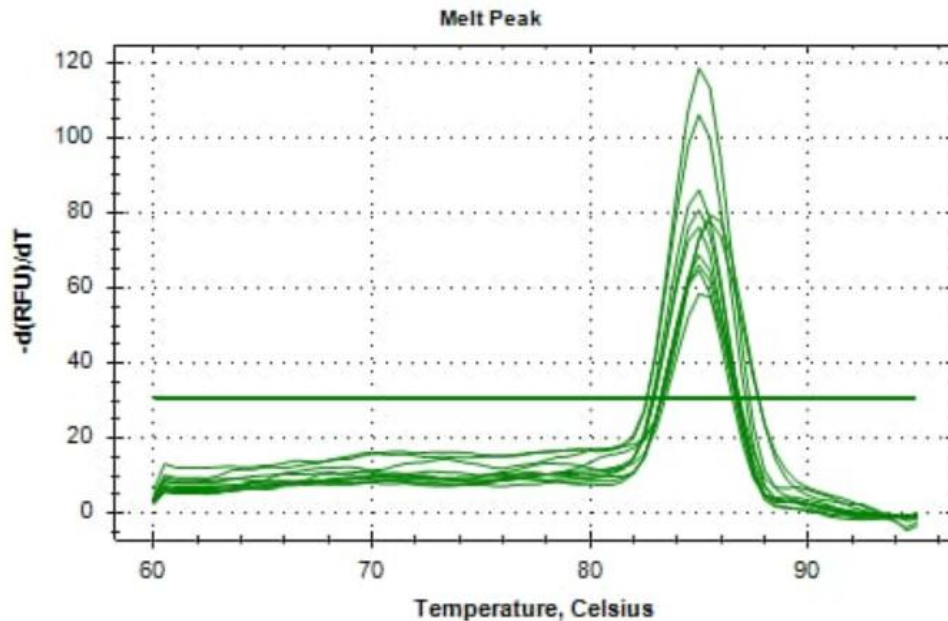


Fig. 5. Melt curve from QPCR of the 16SrRNA gene

The present study identified a significant difference in the 16S rRNA gene sequencing between the groups. The CT values were found to be higher in caries free group, followed by ECC group and S ECC group. CT values are inversely proportional to gene expression. Hence the quantity of *S.mutans* were found to be higher in S ECC group than the ECC and caries free group. The results were similar to the study done by Choi E et al and Veena RL et al, where the authors found an increase in *S.mutans* level in caries active group [42,43]. Similarly, Tanner ACR et al done both microbial and 16SrRNA PCR technique to identify the microbiota of ECC and caries free children. The author identified 74 isolates and he further added that *S.mutans* and *Scardovia wiggsiae* was considered as a caries associated species [44]. On contrary, Ge Y et al and Okada M et al reported an increase in *S.mutans* level in caries free children [38,45].

Moreover, there was a negative correlation between the CT values and dmfs score, lower the CT value, higher the dmfs score. (Figs 1 and 2). As stated previously, CT value is inversely proportional to *S.mutans* count, but it is not necessary that higher dmfs score should have higher level of *S.mutans*. But in the present study, it has been confirmed that, the CT values were negatively correlated with the dmfs score, indicates a higher dmfs score corresponds to an increase in *S.mutans* count.

We expressed the quantity of *S.mutans* in ECC and SECC group in Comparative C_T method. The main advantage of Comparative C_T method is ease of use and the severity can be presented as fold change, which is easy to understand [34]. In the present study, there was a positive correlation of pufa score and *S.mutans* fold change, indicating the severity of *S.mutans* level in ECC and SECC group. However direct comparison of the results of the current study cannot be done with previous studies, as this is one of the first study to compare the pufa score by comparative C_T method. However, comparison with previous study is applicable by comparing the fold change with dmfs score. The results of the present study is consistent with the study done by Choi E et al, where the author found a strong positive correlation to *S.mutans* level and dmfs score [42]. However, the results were contradictory to the study done by Veena RL et al, which showed a weak positive correlation between dmfs score and *S.mutans* level [43].

Real Time PCR is a simple and rapid test with accurate quantification of nuclei acid and greater reproducibility. Real Time PCR monitoring has become a boon to dentistry, as it provides the quantification of bacteria with accurate quantification of individual species, it is easy to correlate with the clinical scenarios. Advantages of Real-Time PCR includes, quick analysis, good

control of quality, Stability of samples over long period of time, the ease of quantification, greater sensitivity, reproducibility and precision, and a lower risk of contamination [46,47].

Melt curve analysis is performed to assess the quality of PCR assays, whether their intercalating dye PCR/qPCR assays have produced single, specific amplicons. As intercalating dyes has the ability to bind to any double-stranded DNA product which are not sequence specific. Hence, melt curve analysis is done to assess the qPCR amplicon length with intercalating dye qPCR assays. In the present study melt curve analysis was done and it showed a single peak, elucidating a pure, single amplicon. (Fig. 5)

Eventhough, ECC is a bacterial-dependent disease, other cariogenic factors such as diet, socioeconomic status and more importantly other micro-organism in saliva could contribute to development of ECC. More research is needed to identify the exact role of synergistic mechanisms of *S.mutans* with mixed microbial environment of oral cavity and to determine the reliability and accuracy in predicting the development of ECC.

Limitation of the study being a cross-sectional study with a smaller sample size. In addition, this study was conducted in children from the district that have similar socioeconomics and demographic information. The result needs to be interpreted carefully. Hence further interventional research is needed in a larger sample size to know the exact expression of 16SrRNA gene before and after intervention

In summary, the use of qRT-PCR to detect and quantify *S. mutans* colonization offers valuable advantages for conducting epidemiological and clinical studies of risk assessment for ECC. A positive correlation between different status of ECC and *S. mutans* colonization in the salivary samples was determined. The study further suggests that children colonized with *S. mutans* are at a higher risk for ECC and it was evident with the pufa score. Utilization of qRT-PCR with species specific primer to *S. mutans* could become more accessible if immediate bacterial cultivation procedure is not possible, hence, it can be used to evaluate *S. mutans* colonization in field study and to assess children's risk for ECC. In addition, this is one of the first study to compare the pufa score of deciduous dentition with the fold change of *S.mutans* by comparative C_T method.

5. CONCLUSION

With the light of available evidence, following conclusion can be drawn

1. There was a higher expression of 16SrRNA gene in SECC group, followed by ECC and caries free group, indicating a high level of *S.mutans* in SECC group
2. There was a negative correlation between dmfs score and CT values
3. There was a positive correlation between pufa score and fold change, indicating an increase in pufa score along with an increase in *S.mutans* level

CONSENT

Informed consent was signed by the parents/Guardians of the children prior to the commencement of the study. Children, whose parents provided consent were included in the study.

ETHICAL APPROVAL

The present study was submitted to and approved by the Scientific Review Board, Saveetha University and ethical approval was obtained from the Institutional Human Ethics Committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Benjamin RM. Oral health: the silent epidemic. Public Health Rep. 2010;125(2): 158-59.
2. Schroth RJ, Brothwell DJ, Moffatt ME. Caregiver knowledge and attitudes of preschool oral health and early childhood caries (ECC). Int. J. Circumpolar Health. 2007;66(2):153-67.
3. Zhu C, Yuan C, Ao S, Shi X, Chen F, Sun X, Zheng S. The predictive potentiality of salivary microbiome for the recurrence of early childhood caries. Front. Cell. Infect. Microbiol. 2018;8: 423.
4. McAuliffe Ú, Kinirons M, Woods N, Harding M, McAuliffe U, Harding M. A retrospective investigation of the oral health records of a cohort of preschool children who received

- extractions under general anaesthesia including cost analysis of treatment. J Ir Dent Assoc. 2017;63(1):38-44.
5. Yin W, Hu DY, Li X et al. The anti-caries efficacy of a dentifrice containing 1.5% arginine and 1450 ppm fluoride as sodium monofluorophosphate assessed using quantitative light-induced fluorescence (QLF). J. Dent. 2013;41:S22-S28.
 6. Casamassimo PS, Thikkurissy S, Edelstein B, Maiorini E. Beyond the dmft: the human and economic cost of early childhood caries. J Am Dent Assoc. 2009;140(6): 650-57.
 7. Becker MR, Paster BJ, Leys EJ et al. Molecular analysis of bacterial species associated with childhood caries. J. Clin. Microbiol. 2002;40(3):1001-1009.
 8. Bighton D, Brailsford S, Samaranayake LP et al. A multi-country comparison of caries-associated microflora in demographically diverse children. Community Dent Health. 2004; 21(1):96-101.
 9. Caufield, PW, Cutter GR, Dasanayake, AP. Initial acquisition of *Mutans Streptococci* by infants: evidence for a discrete window of infectivity. J. Dent. Res. 1993;72(1):37-45.
 10. Mandel ID. Dental caries: although we seem to be paying for the sweet life through the teeth, resistance to caries is possible, both as a natural and an induced phenomenon. AmSci. 1979;67(6):680-688.
 11. Schafer TE, Adair SM. Prevention of dental disease: the role of the pediatrician. Pediatr Clin North Am. 2000;47(5):1021-1042.
 12. Caufield, PW, Griffen AL. Dental caries: an infectious and transmissible disease. Pediatr Clin North Am. 2000;47(5):1001-1019.
 13. Caufield PW, Walker TM. Genetic diversity within *Streptococcus mutans* evident from chromosomal DNA restriction fragment polymorphisms. J. Clin. Microbiol. 1989; 27(2):274-278.
 14. Bigger J. Treatment of Staphylococcal Infections with Penicillin by Intermittent Sterilisation. Lancet. 1944;497-500.
 15. Jayaraman R. Bacterial persistence: some new insights into an old phenomenon. J. Biosci. 2008;33(5):795-805.
 16. Leung V, Lévesque CM. A stress-inducible quorum-sensing peptide mediates the formation of persister cells with noninherited multidrug tolerance. J. Bacteriol. 2012;194(9):2265-2274.
 17. Lewis K. Multidrug tolerance of biofilms and persister cells. Bacterial biofilms. 2008. 107-131.
 18. Wood TK, Knabel SJ, Kwan BW. Bacterial persister cell formation and dormancy. Appl. Environ. Microbiol. 2013;79(23): 7116-7121.
 19. Van Palenstein Helderma WH, Ijsseldijk M, Veld JH. A selective medium for the two major subgroups of the bacterium *Streptococcus mutans* isolated from human dental plaque and saliva. Arch. Oral Biol. 1983;28(7):599-603.
 20. Ravikumar D, Ramani P, Gayathri R. Genotypic diversity of *Streptococcus mutans* in children with and without early childhood caries-A systematic review. J Oral Biol Craniofac Res. 2021;11(2):308-312.
 21. Bighton D, Manji F, Baelum V, Fejerskov O, Johnson NW, Wilton JM. Associations between salivary levels of *Streptococcus mutans*, *Streptococcus sobrinus*, lactobacilli, and caries experience in Kenyan adolescents. J Dent Res. 1989;68:1242-1246.
 22. Hirose H, Hirose K, Isogai E, Miura H, Ueda I. Close association between *Streptococcus sobrinus* in the saliva of young children and smooth-surface caries increment. Caries Res. 1993;27:292-297.
 23. Loesche WJ. Role of *Streptococcus mutans* in human dental decay. Microbiol Rev. 1986;50:353-380.
 24. Kohler B, Andreen I, Jonsson B. The effect of caries-preventive measures in mothers on dental caries and the oral presence of the bacteria *Streptococcus mutans* and lactobacilli in their children. Arch Oral Biol. 1984;29:879-883.
 25. Tankkunnasombut S, Youcharoen K, Wisuttisak W, Vichayanrat S, Tiranathanagul S. Early colonization of mutans streptococci in 2- to 36-month-old Thai children. Pediatr Dent. 2009;31:47-51.
 26. Aas JA, Griffen AL, Dardis SR et al. Bacteria of dental caries in primary and permanent teeth in children and young adults. J. Clin. Microbiol. 2008;46(4):1407-1417.
 27. Becker MR, Paster BJ, Leys EJ, et al. Molecular analysis of bacterial species associated with childhood caries. J. Clin. Microbiol. 2002; 40(3): 1001-1009.

28. Corby PM, Lyons-Weiler J, Bretz WA et al. Microbial risk indicators of early childhood caries. *J. Clin. Microbiol.* 2005; 43(11): 5753-5759.
29. Gross EL, Leys EJ, Gasparovich SR et al. Bacterial 16S sequence analysis of severe caries in young permanent teeth. *J. Clin. Microbiol.* 2010;48(11):4121-4128.
30. American Academy of Pediatrics. Policy on early childhood caries (ECC): classifications, consequences, and preventive strategies. *Pediatr Dent.* 2008; 30(7 Suppl):40-3.
31. WHO. Oral health surveys: basic methods. World Health Organization; France; 2013.
32. Monse B, Heinrich-Weltzien R, Benzian H, Holmgren C, van Palenstein Helderma W. PUFA—An index of clinical consequences of untreated dental caries. *Community Dent. Oral Epidemiol.* 2010;38:77–82.
33. Wu KP, Ke JY, Chung CY et al. Relationship between unstimulated salivary flow rate and saliva composition of healthy children in Taiwan. *Chang Gung Med J.* 2008;31(3):281-6.
34. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C T method. *Nat. Protoc.* 2008; 3(6):1101-1108
35. Loesche WJ. Role of *Streptococcus mutans* in human dental decay. *Microbiol. Rev.* 1986; 50(4):353-380
36. Hajishengallis G, Darveau RP and Curtis MA. The keystone-pathogen hypothesis. *Nat. Rev. Microbiol.* 2012; 10(10): 717-725.
37. Lindquist B, Emilson CG. Distribution and prevalence of *Mutans Streptococci* in the human dentition. *J. Dent. Res.* 1990;69(5): 1160-1166.
38. Ge Y, Caufield PW, Fisch GS, Li, Y. *Streptococcus mutans* and *Streptococcus sanguinis* colonization correlated with caries experience in children *Caries Res.* 2008;42(6):444-448.
39. Jiang S, Gao X, Jin L, Lo E. Salivary microbiome diversity in caries-free and caries-affected children. *Int. J. Mol. Sci.* 2016; 17(12):1978.
40. Ma C, Chen F, Zhang Y et al. Comparison of oral microbial profiles between children with severe early childhood caries and caries-free children using the human oral microbe identification microarray. *PLoS One.* 2015;10(3):0122075.
41. Nurelhuda, NM, Al-Haroni M, Trovik TA, Bakken V. Caries experience and quantification of *Streptococcus mutans* and *Streptococcus sobrinus* in saliva of Sudanese schoolchildren. *Caries Res.* 2010;44(4):402-407.
42. Choi EJ, Lee SH, Kim YJ. Quantitative real-time polymerase chain reaction for *Streptococcus mutans* and *Streptococcus sobrinus* in dental plaque samples and its association with early childhood caries. *Int J Paediatr Dent.* 2009;19(2):141-147.
43. Veena RL and Nagarathna C. Correlation of *streptococcus mutans* and *streptococcus sobrinus* colonization with and without caries experience in preschool children. *Indian J Dent Res.* 2020; 31(1): 73-79.
44. Tanner ACR, Mathney JMJ, Kent RL, et al. Cultivable anaerobic microbiota of severe early childhood caries. *J. Clin. Microbiol.* 2011;49(4):1464-1474.
45. Okada M, Soda Y, Hayashi F, et al. PCR detection of *Streptococcus mutans* and *S. sobrinus* in dental plaque samples from Japanese pre-school children. *J. Med. Microbiol.* 2002;51(5):443-447.
46. Morillo JM, Lau L, Sanz M, Herrera D, Silva A. Quantitative real-time PCR based on single copy gene sequence for detection of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*. *J. Periodontal Res.* 2003;38(5):518-524.
47. Novais CM, Pires-Alves M, Silva FF. PCR tempo real. *Rev. Biotecnol. Cienc. Des.* 2004:33: 11.

© 2021 Ravikumar et al.; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Peer-review history:
 The peer review history for this paper can be accessed here:
<https://www.sdiarticle4.com/review-history/72581>