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

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Authors’ contributions

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

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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 pufo 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.

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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 etiology 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

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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 score 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.
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 into 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 45º 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 CT 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+/铜ere 1.93, Group II (ECC), the mean CT values were 19.93+/铜ere 0.88 and Group III (SECC), it was found to be 19.41+/铜ere 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)

| 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 show 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 depict 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 in 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

| 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
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).

![Correlation of pufa score with the fold change in children with ECC](image1)

$y = 0.5808x - 1.6418$
$R^2 = 0.5962$

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

![Correlation of pufa score with the fold change in children with S ECC](image2)

$y = 0.7836x - 3.6698$
$R^2 = 0.733$

**Fig. 4. Correlation of pufa score with the fold change in children with S ECC**
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 SECC group. CT values are inversely proportional to gene expression. Hence the quantity of \textit{S. mutans} were found to be higher in SECC 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 \textit{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 \textit{S. mutans} and \textit{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 \textit{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 \textit{S. mutans} count, but it is not necessary that higher dmfs score should have higher level of \textit{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 \textit{S. mutans} count.

We expressed the quantity of \textit{S. mutans} in ECC and SECC group in Comparative C\textsubscript{T} method. The main advantage of Comparative C\textsubscript{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 \textit{S. mutans} fold change, indicating the severity of \textit{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\textsubscript{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 \textit{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 \textit{S. mutans} level [43].

Real Time PCR is a simple and rapid test with accurate quantification of nucleic 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<sub>T</sub> 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.

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