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Detection of Streptococcus Mutans from Human Saliva Using 16SrRNA and spaP Genes in Regard with Dental Caries

M. Sameer Kadhim (PhD)[*1], A. Taha Mohammed (PhD)[2]

1.Department of Pedodontics and Preventive Dentistry, Al-Rafidain University College, Baghdad, Iraq.

2.Department of Pedodontics and Preventive Dentistry, College of Dentistry, University of Baghdad, Baghdad, Iraq.

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ABSTRACT

Background and Objective: Dental caries is the most common disease worldwide, and is a major healthcare problem. Streptococcus mutans is considered as the main factor causing dental caries. This study was conducted to assess Streptococcus mutans by its 16S rRNA gene and adhesin gene (spaP) as an early predictor of caries development.

Methods: This case-control study was conducted on 80 Iraqi pre-school children aged 3-5 years old. Subjects were divided into two groups: the case group, which included 40 children with severe caries, and the control group, which included 40 caries-free children. A dental examination was performed by diagnostic criteria of World Health Organization (WHO). Unstimulated salivary samples were taken from all children, and DNA of Streptococcus mutans was extracted from the all saliva samples. The 16S rRNA and spaP genes were detected using polymerase chain reaction (PCR), and their presence was compared to dental caries severity.

Findings: The age and gender of the children had no significant effect in this study. The prevalence of the Streptococcus mutans 16SrRNA gene was 98.8% positive and 1.2% negative in all study groups. Regarding spaP, a significant difference discovered between the case (92.5%) and control (50%) groups ($p \leq 0.05$).

Conclusion: The results showed that the presence of spaP gene of Streptococcus Mutans can predict caries development. Therefore, more dental care is emphasized in these people.

Keywords: *Streptococcus Mutans, 16SrRNA, SpaP, Virulence Genes, Dental Caries.*

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[*]Corresponding Author: M. Sameer Kadhim (PhD)
Address: Department of Pedodontics and Preventive Dentistry, Al-Rafidain University College, Baghdad, Iraq.
Tel: +964 (772) 1014811. **E-mail:** maitha.alnasri@ruc.edu.iq

Introduction

Dental caries is one the most prevalent childhood problems (1); people are subjected to dental caries throughout their lifetime (2, 3). It is considered as a multifactorial disease depending on the interaction of several factors, acidogenic bacteria, fermentable carbohydrate, host and time (4-6). Dental caries develops as a dynamic process of demineralization of dental hard tissues by bacterial metabolic products. Streptococcus mutans is considered as the main cause of dental caries (7, 8). These bacteria produce organic acids as a by-product of their metabolism of fermentable carbohydrates. Streptococcus mutans thrives in acidic environments, eventually becoming the dominant bacterium in cultures with permanently reduced pH (9, 10). Different methods were used to detect putative pathogen, which included cultivation; direct microscopy; enzyme tests; enzyme-linked immunosorbent assays and species-specific DNA probes (11, 12). Polymerase chain reaction (PCR) methodology is now widely accepted when compared to conventional culture techniques, and provides a more sensitive means of detection of putative bacteria species (13-15). In PCR technique, specific primers based on the 16S rRNA gene sequences were used to detect S. mutans (16, 17).

S. mutans is a causative factor of dental caries in humans (18). The bacterium's capability to form dental biofilm on tooth surfaces is an important virulence property (19). Furthermore, this bacterium has the ability to produce multiple glucan-binding proteins, glucosyltransferases and collagen-binding protein, all of which work together to form dental plaque and cause dental caries (10). Streptococcus mutans has virulence genes involved in adhesion of bacteria: gbps (gbpA, gbpB, and gbpC.) and spaP. SpaP is antigen of cell surface encoded by the spaP gene (20). It is also known as Ag I/II; Pac; AgB; Pl; Sr; SpaA; Pag; SspA; SspB; and SoaA. This surface fibrillar adhesin attaches to bacteria to salivary agglutinin glycoprotein and proline-rich protein of the acquired pellicle on the tooth surface (20, 21). After irreversible adhesion, oral bacterial growth, division, and colonization occur (22). An interaction between the adhesive and the substrate is observed in the second mechanism of adhesion (sucrose-independent) (18, 23). This study aims to identify S. mutans bacteria directly from saliva using PCR techniques, while targeting the 16S rRNA and spaP genes and by comparing their presence with the dental caries among Iraqi preschool children.

Methods

This case-control study was conducted in College of Dentistry, University of Baghdad, Baghdad, Iraq. Ethical committee approval was obtained from the College of Dentistry, University of Baghdad for the protocol before processing the study (Approval reference number: 365). To obtain permission for the children's participation in the study, special consent was distributed among their parents. Only children with signed consent were involved in this study and they were investigated according to inclusion and exclusion criteria. The total sample size was 80 children, they were 3-5 years old: 40 children with severe caries (case group) and 40 children were caries-free (control group). The participants were chosen from the College of Dentistry, University of Baghdad and Al-Rafidain University College of dentistry, Baghdad, Iraq. Sample size was determined Using G Power 3.1.9.7 software "http://www.gpower.hhu.de/" with a power of 80%, two-sided alpha error probability of 0.05, and Cohen's effect size of 0.8. The samples were selected randomly, and both groups were matched for age and gender.

Evaluation of oral conditions: Examination of dental caries was done using dmft and dmfs index according to WHO criteria (24). The examination was done while the child was seated on a dental chair. The following conditions were used to exclude children from the study: children without their parents' consent, children suffering from systemic diseases, children who had fluoride supplements or fissure sealant, children who

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were on medicine (antibiotic or otherwise) during the saliva collection time, children who ate one hour before the collection of the sample, children with mild or moderate dental caries and uncooperative, fearful and anxious children. They were classified according to their caries experience: Those with dmft=0 were considered as caries-free and those with dmft greater than or equal to 6 as severe caries (dmft≥6) according to Drury et al. (25).

Sample collection: Unstimulated salivary sample collection was done according to Khurshid et al. (26). The child had to refrain from drinking, eating and performing oral hygiene procedures for at least one hour prior to saliva collection, which was performed between 8 and 11 a.m. All samples were stored by freezing at (-20 °C), while transported to the laboratory for molecular analysis.

DNA extraction and the detection of streptococcus mutans via polymerase chain reaction: DNA of S.mutans bacteria was extracted according to instructions of Bacterium Genomic DNA isolation kit (Norgen®, Canda). Through downloading different Streptococcus Mutans genomes from GenBank as FASTA files, the primers of this study have been used as a bioinformatics software tool for verifying each pair of primers' binding site and appropriate annealing temperature (Table 1).

Table 1. Primers

Name of primer	Primer sequence	Size of product
16SrRNA	F "`5 -CCACACTGGGACTGAGACAC-3" R (3- GTTTACGGCGTGGACTACCA-`5)	507 pb
SpaP	F "`5-AAC GAC CGC TCT TCA GCA GAT ACC -3" R "3- AGA AAG AAC ATC TCT AAT TTC TTG`-5"	192 pb

F: Forward, R: Reverse

Molecular detection of 16S rRNA and spaP gene: The reaction was carried out using the best PCR conditions for each gene, as shown in Table 2. Electrophoresis in 2% agarose gel was used to separate the amplicons. Ten microliters of PCR product and DNA ladder were loaded into the gel wells. For 80 minutes, the power supply voltage was mostly held constant at 80V. Gel documentation with a high-resolution camera was used at the end of the run to capture images and analyze the bands. Bands with 507bp represent 16srRNA gene while bands with 192bp represent spaP gene.

Table 2. PCR conditions for genes

Cycle No.	Stages	Temperature	Time
1	"Initial Denaturation"	94 ºC	5 min
38x	"Denaturation"	94 ºC	30 sec
	"Annealing"	57 ºC	45 sec
	Extension	72 ºC	45 sec
1	Final Extension	72 ºC	7 min

All the results were expressed as Mean±SD, SE and Mean difference. Significance levels: probability of error can be tested as (p-value): Non-significant $p>0.05$, Significant $p\leq0.05$. Unpaired-Sample T-Test was employed to evaluate the presence of significant differences. Logistic regression was used for adjusting odds ratio. Chi-square test was used to assess the categorially association variables and genetic association.

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Results

The age and gender of the children in the sample was approximately normally distributed and ranged from 3-5 years old, with a mean of 4.13 and a standard deviation (SD) of ±0.791 years. "No-significant differences" were found between the study groups regarding age and gender, as shown in Table 3. The control group consisted of caries-free children with dmf=0 and children with severe caries consisted a case group, where the mean dmft was 11.80±2.588 and the mean dmfs was 28.80±19.435.

Genes: No-significant difference was identified regarding 16srRNA between groups, while regarding the adhesion gene spaP, significant difference ($p \leq 0.05$) was detected between groups. spaP gene was positive in 92.5% of study group as compared to 50% of controls. As shown in Table 4: Odd ratio was 12.33 and confidence interval was 3.262-46.625 (Table 5). PCR result for 16srRNA samples in gel electrophoresis was illustrated in Figures 1 and 2. The result of PCR for samples in gel electrophoresis was illustrated in Figure 3 and figure 4 as it was best at 192 bp.

Dental caries experience and its association with spaP gene: Regarding bacterial gene 16sr RNA and its association with dental caries in all cases in case group have positive gene detection which is incomparable with dental experience statistically; only bacterial adhesin gene (spaP) was compared and it showed a significant difference concerning caries experience ($p \leq 0.05$) (Table 6).

Table 3. Baseline characteristics of sample

Status	Number	Mean±SD	Standard Error	Mean difference	T-test ^	p-value
Age						
Control	40	4.13±0.791	0.125	0.000	0.000	1.000*
Case	40	4.13±0.791				

^unpaired T-test, *non-significant

Table 4. Distribution of bacterial gene between study groups

Bacterial geneticdetection	Study group		Total (n=80) Number (%)	p-value
	Case (n=40) Number (%)	Control (n=40) Number (%)		
16srRNA				
Positive	40(100)	39(97.5%)	79(98.8)	0.314
Negative	0(0)	1(2.5%)	1(1.2)	
spaP				
Positive	37(92.5)	20(50.0)	57(71.3)	0.000*
Negative	3(7.5)	20(50.0)	23(28.7)	

***Significant p≤0.05**

Table 5. Analytic statistics of spaP gene among study groups

	Study sample (n=80)		Fisher test (p-value)	OR	CI 95%
	Control	Case			
spaP					
Negative	20	3	0.000*	12.33	3.262-46.625
Positive	20	37			
Total	40	40			

Degree of freedom=1, *significant p-value≤0.05

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Table 6. Dental caries and bacterial adhesine gene (spaP) of case group

Caries experience	(spaP) gene (n=40)		Mean differences	T-test	p-value*
	Positive (n=37) Mean±SD	Negative (n=3) Mean±SD			
dmft	12.41±3.531	9.67±2.082	-2.739	-1.315	0.0196
dmfs	31.65±18.476	15.67±7.638	-15.982	-1.474	0.0149

df: 38, *Significant p≤0.05



Figure 1. Electrophoresis of 16srRNA for caries free children



Figure 2. Electrophoresis of 16srRNA gene for children with severe caries

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Figure 3. Electrophoresis of spaP gene for caries-free children



Figure 4. Electrophoresis of spaP gene for children with severe caries

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Discussion

The PCR method was used to detect S. mutans directly from saliva with constant part of 16S rRNA gene primer and this result was similar to other studies (27, 28). The 16S rRNA gene was designed specifically for S.mutans, eliminating the need for another primer to detect cariogenic S. mutans (29). The result of current study indicated S. mutans bacteria were present in all saliva samples (case and control groups). Previous studies indicated that the microbiota composition did not differ between caries and caries-free groups which is in agreement with present study (19, 30). These can prove the theory "S. mutans is a normal resident of human oral cavity"(31). Streptococcus mutans is one of the major etiologic agents of dental caries development (32). Nevertheless, there were children had S. mutans but with no caries development, so the result of this study is in coordination with the other studies (33, 34), which can explain "The most recurrent difference between those with dental caries and those who have maintained their caries-free status is related to the management of plaque pH; Subjects with no dental cavities appear to neutralize plaque acids more successfully than those with dental caries."(2, 31). However, diseases can occur only if there is an imbalance in metabolic and composition of dental biofilm microbial (35). Aside from this fact, multiple laboratory investigations have demonstrated that S. mutans may change the local environment by establishing a rich and low pH, providing a suitable niche for other acidogenic and aciduric species to thrive. This suggests that S. mutans is not the only factor in the formation of dental caries (7). Lactate production is clearly important in caries-associated ecosystems (30), and caries-associated biofilms frequently contain a high number of bacteria that use organic acids as an energy source (36). The findings of this study showed that the prevalence of S. mutans $spaP^{+}$ gene was higher in children with severe caries than in children without caries, with a significant difference and this result was in agreement with previous studies (20, 37, 38), in which the scientists found a substantial correlation between the high frequency of the S. mutans, spaP gene and the prevalence of caries in preschoolers. The organization, structure, and interaction of the S. mutans adhesin gene spaP with its salivary agglutinin receptors have been investigated (36).

As reported in this study, some caries-free children, despite having spaP gene, showed no dental caries and that may be elucidated by spaP adhesin harboring a variants genotypes; Despite equal levels of SpaP expression, A, B, and C (also known as A1, B2 and B2) can modify binding levels (39). The interaction of spaP with salivary receptors is dependent on strain of the S. mutans (40).

Streptococci mutans bacteria were found in all samples according to the 16srRNA gene. A relationship was discovered between childhood caries and the presence of bacteria with the adhesin gene spaP positive. Thus, according to this study, spaP gene can be used in prediction for caries development in young children.

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