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Detection of S. *mutans* and S. *sobrinus* from Dental Plaques Adhered on Zirconia, PFM and Ni-Cr Crown by RT-PCR

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Abstract: ***The purposes of this studywas to detect S. mutans and S. sobrinus from Dental Plaques adhered on Zirconia, PFM and Ni-Cr crown by RT-PCR amplification. Supragingival and the subgingival plaque was taken from the crown site of the 118 participants from all the three groups who had crown restoration and were categorized as Group I (Zirconia) (27 cases), Group II(PFM) (49 cases) and Group III(Ni-Cr) (42 case), respectively by using sterile curettes (Gracey Curettes). The PCR amplified products were subjected to electrophoresis in agarose gel and visualized on a UV transilluminator. Patient's (118) age ranged between 18 to 40 years divided into the three groups who had crown restoration and were categorized as Group I (Zirconia) (27 cases), Group II(PFM) (49 cases) and Group III(Ni-Cr) (42 case), respectively. The S. mutans and S. sobrinuswas found to be present in 60.2% and 44.9 % of cases respectively. In the present study, S. sobrinus alone was present only in 35.6% of cases. A significant finding of the present study was presence of both S. mutans and S. sobrinus in 24.6% of cases and 19.5% were negative for both.Genomic DNA (gDNA) extracted from S. mutans was analyzed on 1% agarose gel electrophoresis and found to be good quality and quantity off or further use. Total gDNA isolated from dental plaque underwent PCR amplification using primer pair specific to 16S rDNA of the genus Streptococci. One percent agarose gel electrophoresis depicts the positive PCR amplification at 712 bp when compared with standard 1kbp. Another PCR reaction was set using gtfB primers specific for S. mutans for functional gene identification. 1.2% agarose gel electrophoresis was done and a positive amplication was observed at 517 bp when compared to 1000 bp standards, depicting the presence of functional genes (gtf) in S. mutans. Among the mutans streptococci group, S. mutans and S. sobrinus are the most frequently isolated species from human dental plaque and are believed to be the major aetiological agents for tooth decay.***

Keywords: Dental Plaques; Streptococcus Mutans; Streptococcus Sobrinus; Polymerase Chain Reaction; Crown

1. Introduction

Dental caries (tooth decay) is a chronic disease, caused by acidogenic bacteria, may lead to dissolution of enamel and dentin, (coronal caries) and cementum and dentin (root caries) (1).Streptococcus *mutans* and Streptococcus *sobrinus* are powerfullycompromised in the onset of human dental caries. Many studies have shown the relationship of *S. mutans* and *S. sobrinus* with higher caries levels (2-4) and many studies have found that *S. sobrinus* occurrence is more strongly related with high caries activity than that of *S. mutans* (5-6). It is considered that determination of these two species in early childhood could be important for diagnosing and preventing dental caries.

Various authors (7-8) have developed PCR methods to detect S. *mutans* in samples of dental plaque. To facilitate biological sample collection for epidemiological studies, it has been developed a PCR method to detect *S. mutans* and *S. sobrinus* in saliva, using specific primers for the genes that encode glucosyltransferase (gtfB in *S. mutans* and gtfI in *S. sobrinus*), which can be used to assess the prevalence of these organisms in epidemiological studies (9).

Based on a strong relationship between microorganism counts in saliva and plaque, *S. mutans* determination in saliva has been suggested as a suitable method for identifying patients at high risk of dental caries (10). As saliva is continuously in contact with all the teeth, it provides a better reflection of the colonization of mutans streptococci on all dentition (11).

The purposes of this study was to detect S. mutans and S. *sobrinus* from Dental Plaques adhered on Zirconia, PFM and Ni-Cr crown by RT-PCR amplification.

2. Material and Methods

Plaque Sampling

Supragingival and the subgingival plaque was taken from the crown site of the 118 participants from all the three groups who had crown restoration and were categorized as

Group I (Zirconia) (27 cases), Group II(PFM) (49 cases) and Group III(Ni-Cr) (42 case), respectively by using sterile curettes (Gracey Curettes). Dental examinations were performed under natural light, using a plane dental mirror and explorer. The plaque samples were suspended in 1 ml of sterile Phosphate buffer saline PBS (0.12 M NaCl, 0.01 M Na_2HPO_4, 5mM KH_2PO_4 pH 7.5). The samples were transported on icebox to the laboratory until processed within 24 hours.

GenomicDNAExtractionfromPlaqueSamples

Phenol: chloroform method was used for extraction of genomic DNA according to Paster *et. al.* (16) with few modifications as 100 µL of plaque sample transferred to a new sterile micro centrifuge tube where 100 µL of the lysis buffer was added (50 mM tris buffer, 1 mM EDTA, 0.5% Tween 20, and proteinase K (200µg/ml). pH 8), then incubation at 55-60 ºC was done for 1.5 hour. Inactivation of proteinase K was done at 90 ºC for 5 minutes. After that, samples were cooled on ice for few minutes and the DNA was precipitated by the addition of an equal volume of ice-cold isopropanol and incubated at refrigerator overnight. The DNA pellet was washed with 70% ethanol, and was then rehydrated in 20- 30 µl TE buffer (10 mM Tris- HCl, and 1 mM EDTA) (pH 8) (16).

RT-PCR

Oligonucleotide primers F5'-ACTACACTTTCGGGTGGCTTGG and R5'-CAGTATAAGCGCCAGTTTCATC were designated to amplify a 517bp-DNA fragment of the gtf B sequence of S. *mutans*, and primers F5'-GATAACTACCTGACAGCTGACT and R5'-AAGCTGCCTTAAGGTAATCACT were designated to amplify a 712 bp-DNA fragment of the gtf I sequence of S. sobrinus. The PCR mixture was the same used by Oho et al (7). The PCR conditions were denaturation at 95°C for 30 s, followed by annealing at 59°C for 30 s, and extension at 72°C for 1 min. This amplification was repeated for 30 cycles. The final cycle was run at 72°C for 5 min. Along with these samples, positive and negative controls were run for each experiment, using purified genomic DNA from S. *mutans* and S. *sobrinus* respectively, as positive controls, and distilled water as negative controls. PCR products were subjected to run gel electrophoresis.

Gel Electrophoresis

The PCR amplified products were subjected to electrophoresis in agarose gel dissolved in 1X TBE buffer, 0.89 M Boric Acid, 20 mM EDTA. 5 µL of PCR product was mixed with 4µL of loading dye and then loaded into the well of the gelalong with 100-bp DNA ladder marker. PCR products were separated through the gel at an electric current of 80 V for 1 hour by using horizontal gel electrophoresis apparatus. Gels were stained with ethidium bromide (0.5µg/ml) and visualized on a UV transilluminator.

Statistical Analysis

Categorical variables will be presented in number and percentage (%) and continuous variables will be presented as mean and SD. Quantitative variables will be compared using one-way ANOVA between three groups. A *p* value of <0.05 will be considered statistically significant. The data will be entered in MS EXCEL spreadsheet and analysis will be done using Statistical Package for Social Sciences (SPSS) version 21.0.

3. Results

Table 1: Percentage distribution of patients according to their gender in all three groups

Gender	Group I (Zirconia) (N=27)	Group II (PFM) (N=49)	Group III (Ni-Cr crown) (N=42)	Total (N=118)
Male	11(40.7)	29(59.2)	25(59.5)	65(55.1)
Female	16(59.3)	20(40.8)	17(40.5)	53(44.9)

Table 1 shows Percentage distribution of patients according to their gender in samples of all three groups.

Table 2: Age distribution: (18 to 40 years)

	Group I (Zirconia) (N=27) Mean±SD	Group II (PFM) (N=49) Mean±SD	Group III (Ni-Cr crown) (N=42) Mean±SD	P value
Age	25.0±2.63	38.3±3.45	35.2±3.12	0.832

Applied one-way ANOVA for significance. Consider not significant.

Patient's age ranged between 18 to 40 years. The one-way analysis of variance showed that there is no significant difference between groups mean age, which is an evidence to discard age effects on the processes of bacterial accumulation.

Table 3: Distribution of S.*mutans*and S.*sobrinus* in different base metal alloy groups. (N=118)

Organism Present		Group I (Zirconia) (N=27)	Group II (PFM) (N=49)	GroupIII (Ni-Cr crown) (N=42)	Total (N=118)
S. *mutans*	S. *sobrinus*				
+	-	10(37.0)	17(34.7)	15(35.7)	42(35.6)
+	+	5(18.5)	13(26.5)	11(26.2)	29(24.6)
-	+	3(11.1)	11(22.4)	10(23.8)	24(20.3)
-	-	9(33.4)	8(16.4)	6(14.3)	23(19.5)

The S. *mutans* and S.*sobrinus*was found to be present in 60.2% and 44.9% of cases respectively. In the present study, S. *mutans*alone was present only in 35.6% of cases where as S. *sobrinus* alone was present only in 20.3% of cases A significant finding of the present study was presence of both S. *mutans* and S. *sobrinus* in 24.6% of cases and 19.5% were negative for both (Table-3).

Table 4: Oligonucleotidesequences, nucleotide (bp), used in this study forS. *mutans* and S. *sobrinus*

Bacterial species	Targeted gene	Oligonucleotide sequence (5′-3′)	Nucleotide (bp)	location	Reference
S.*mutans*	GTFB	(F) ACTACACTTTCGGGTGGCTTGG	517	793–814	*Oho T,etal*., 2000
S.*mutans*	GTFB	(R)CAGTATAAGCGCCAGTTTCATC	517	1288–1309	
S. sobrinus	GTFI	(F) GATAACTACCTGACAGCTGACT	712	871–892	
S. sobrinus	GTFI	(R) AAGCTGCCTTAAGGTAATCACT	712	1561–1582	

Genomic DNA (gDNA) extracted from *S. mutans* was analyzed on 1% agarose gel electrophoresis and found to be good quality and quantity off or further use. Total gDNA isolated from dental plaque underwent PCR amplification using primer pair specific to 16S rDNA of the genus Streptococci [Figure 1]. One percent agarose gel electrophoresis depicts the positive PCR amplification at 712 bp when compared with standard 1kbp. Another PCR reaction was set using *gtf*B primers specific for S. *mutans* for functional gene identification. 1.2% agarose gel electrophoresis was done and a positive amplication was observed at 517 bp when compared to 1000 bp standards, depicting the presence of functional genes (*gtf*) in *S. mutans*.

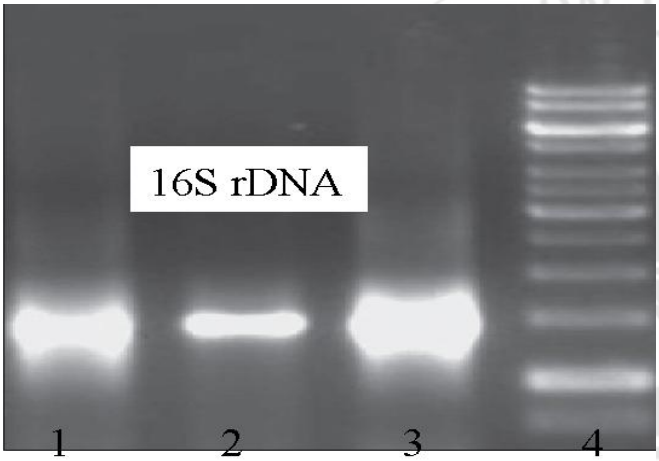

Figure 1: Amplification of 16S gene using S. *mutans* DNA (gel picture)

4. Discussion

Dental caries is a multifactorial infectious disease and is still considered as one of the most prevalent biofilm-mediated diseases affecting humans. The most important risk factor in any disease is the causative agent.

Among the mutans streptococci group, S. *mutans* and S. *sobrinus* are the most frequently isolated species from human dental plaque and are believed to be the major aetiological agents for tooth decay. These micro-organisms have the capability of adhering to the enamel surface and forming a bio-film facilitated by extracellular polysaccharides produced by using sugars in the diet as a substrate, which favors demineralization measured by the acid products from bacterial metabolism (12).

Patient's (118) age ranged between 18 to 40 years divided into the three groups who had crown restoration and were categorized as Group I (Zirconia) (27 cases), Group II(PFM) (49 cases) and Group III(Ni-Cr) (42 case), respectively.Zirconia crown is less used because of its higher price in comparison with other two PFM and Ni-Cr crowns (17).The one-way analysis of variance showed that there is no significant difference between groups mean age, which is an evidence to discard age effects on the processes of bacterial accumulation. Abbas et al. (18) also outlined in their experimental study carried out in Anbar province, Iraq that age had no effects on the processes of bacterial accumulation.

In the present study, dental plaque adhered on Zirconia, PFM and Ni-Cr crown was used for the detection of cariogenic micro-organisms rather than saliva. Various previous studies have used saliva for detecting cariogenic bacteria; however, the tendency in recent studies has been towards the use of dental plaque. As the intention was to relate the presence of cariogenic bacteria and dental caries, it was found that using saliva as a source of cariogenic bacteria does not permit establishing an effective association. Although the presence of S. *mutans* is high in saliva, it is lower on the surface of enamel, where this bacterium actually manifests its capacity to produce acids and result in subsequent demineralization (12).

The S. *mutans* and S. *sobrinus* was found to be present in 60.2% and 44.9 % of cases respectively. In the present study, S. *sobrinus* alone was present only in 20.3% of cases. However, in a previous study by Nurelhuda NM et al., [15], A significant finding of the present study was presence of both S. *mutans* and S. *sobrinus* in 24.6% of cases and 19.5% were negative for both. Thus, the presence of both the bacteria together can be considered an important pathogenic factor in the development of carious lesions.

In present study, *gtf*B was targeted as a functional gene, PCR amplification was done both with positive and negative controls with species-specific *gtf* primers. The amplification observed in 1.2% agarose gel electrophoresis depicts the presence of *gtf*B genes, hence confirming the presence of *S. mutans* in the dental plaque samples. Moreover, the amount of bacterial load can also be determined using real time PCR.

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