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Detection of *Streptococcus mutans* and *Streptococcus sobrinus* in Human Dental Plaque Samples by Using Semi-Quantitative Real-Time Polymerase Chain Reaction

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Dental caries, a highly prevalent infectious disease in humans is caused by the bacterial plaque that coats the teeth surface and is a serious public health concern. Recently, the formation of dental plaque has been associated with the occurrence of various other systemic diseases, Alzheimer's disease, Cardiovascular diseases, Rheumatoid Arthritis, Respiratory diseases, Bacteremia and Cancer. Despite the fact that both Streptococcus mutans and Streptococcus sobrinus are the major etiologic agents of dental caries, S. mutans is more prevalent than S. sobrinus in dental plaque. Early detection of S. mutans and S. sobrinus was carried out from five caries affected dental plaque samples collected from Sri Ramakrishna Dental College and Hospital, Coimbatore, by using semi-quantitative real-time PCR. Specific primers for gtfB and gtfI genes of S. mutans and S. sobrinus respectively were used for the quantification of cariogenic bacteria in the given dental plaque samples. The Biopro Oral Microbiome transport media was prepared to carry dental plaque samples from the hospital to the laboratory. Genomic DNA extraction was done by employing magnetic beads and spin columns provided in the Biopro DNA isolation kit. Various biochemical tests were performed on the bacterial cultures isolated from dental plaque.

Keywords: Dental caries; Dental plaque; Polymerase Chain Reaction; *Streptococcus mutans; Streptococcus sobrinus.*

The "Microbiome" found in a healthy human body comprises of 3.8 x 10^{13} bacterial cells and other microorganisms[1]. One of the body's most complex microbiomes is found in the oral cavity, and oral bacteria plays a major role in the development and occurrence of Dental Caries (tooth decay)[2]. Dental caries is a commonly occurring chronic illness that develops as a result of the acidic demineralization of tooth tissues brought about by bacterial fermentation of dietary carbohydrates[3]. Around the world, 3.5 billion people suffer from oral diseases, 2 billion people are thought to have Dental Caries, and 514 million children have primary tooth decay[4].

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Dental plaque consists of a physically and functionally organized, species-rich microbial community[5]. Approximately 80% of the microorganisms of the biofilm is dominated by Streptococcus which has a role in the initial colonization process, followed by Actinomyces and other bacteria[6]. During high availability of fermentation substrates, Streptococcus species has the ability to ferment carbohydrates into acetic, formic and lactic acid which in turn reduces pH values near five[7]. Many strong acid-producing and acid-tolerant organisms, including *S. mutans*, interact with other biofilm residents and are the principal causal agents of dental caries[8,9]. *S. mutans,* a gram-positive facultative anaerobic bacteria, is typically found in biofilms on tooth surfaces, or in dental plaque[10].

The two Steptococcus species, *S. mutans* and *S. sobrinus* typically co-exist in a person independently, with *S. mutans* being more common in areas around caries and having a close association with the disease condition[11]. When cultivated on plates using Mitis salivarius agar, a selective medium for *S. mutans*, *S. mutans* colony morphology is rough, whereas *S. sobrinus* is smooth[12]. Another important characteristic that explains why *S. mutans* is the main causative agent of dental caries is due to its ability to adapt to abrupt and significant environmental changes inside the tooth plaque[13]. *S. mutans* can withstand frequent and quick environmental changes such as nutrition availability, aerobic to anaerobic transitions, and pH shifts because it uses carbohydrates to create biofilm on tooth surfaces[14].

Despite the fact that caries is a multi-microbial illness, targeting *S. mutans* specifically in dental biofilms is thought to be an effective strategy for its prevention[13]. This is primarily due to the fact that *S. mutans* GTFs (glucosyltransferases), particularly water-insoluble glucan-synthesizing GTFs, are associated with the bacteria's cariogenicity[10]. The water-insoluble glucans (also called mutans), are rich in alpha (1'!3) linkages produced by *gtfB* gene of *S. mutans*, are essential for the development of a stable biofilm matrix[13,15]. This matrix in turn promotes bacterial colonization on the tooth surface and also acts as a diffusion barrier, maintaining the acidic environment necessary for cariogenic bacteria to flourish[16]. Both the *S. mutans gtfB* gene and the *S. sobrinus gtfI* gene encode for glucosyltransferase enzyme[10].

A semi-quantitative real-time PCR reaction was performed to detect 16S rRNA in the genomic DNA purified from dental plaque samples in order to confirm the presence of bacteria in the dental plaque samples. In the current study the presence of *S. mutans* and *S. sobrinus* was confirmed in the subgingival plaque samples by using species specific primers of glucosyltransferase gene and real-time polymerase chain reaction for detection.

MATERIALS AND METHODS

Collection of sample

The clinical dental plaque samples were aseptically collected from the subgingival region of five randomly selected patients visiting Sri Ramakrishna Dental College & Hospital, Coimbatore. The dental plaque samples were scraped from the tooth surface of the patients using a dental explorer, collected in sterile swabs attached to toothpicks and were immediately dipped in Oral Microbiome transport media. Collection tubes were sealed with parafilm to prevent contamination during transportation and were stored at 4!.

Preparation of Oral Microbiome transport medium

The Biopro Oral microbiome transport medium (50 ml) was prepared at Alchem Diagnostics, Coimbatore for carrying dental plaque samples. This Oral microbiome transport medium consists of modified Hanks basal salt solution and a buffer to maintain the pH at 7.3 +/- 0.2. The medium also includes protein for stabilisation. It helps store samples for long periods by preserving bacteria when stored frozen in the presence of a cryoprotectant. The Oral microbiome transport medium was sterilized using 0.22 µm PES filter and stored at temperatures between 10 to 30!.

Preparation of Genomic DNA from plaque sample

The dental plaque samples dissolved in 200µL of oral microbiome transport medium were harvested by centrifugation at 5000 x g for 10 minutes in order to obtain the cell pellet. The genomic DNA was isolated from the cell pellet and supernatant was discarded. Bacterial

genomic DNA extraction was carried out from the five dental plaque samples by using Biopro DNA extraction kit. The protocol from the manufacturer's instruction manual was followed except for the inclusion of an RNase treatment step. The Biopro DNA Extraction kit provides a simple, convenient and reliable protocol for the isolation of high quality DNA using unique buffers. The basic four steps used in the extraction of DNA by using the Biopro DNA extraction kit are: Lysis, Binding, Washing and Elution. The kit was used to isolate DNA either by employing magnetic beads or spin column.

Agarose gel electrophoresis

The concentrations of DNA purified by using Biopro magnetic beads and spin columns were determined by using the Nanodrop Spectrophotometer (Thermo Scientific, USA). The Absorbance at 260 nm of the DNA samples was measured and the concentrations calculated. The Absorbance of the DNA samples were also measured at 280 nm in order to estimate the purity

Table 1. Primers designed for semi-quantitative RT-PCR reaction. Sequence of 16S rRNA universal primer, *S. mutans gtfB* and *S. sobrinus gtfI* gene primers

Target gene	Primer	Primer sequence (5"! 3')	Amplicon length (bp)	References
16S rRNA (Universal primer)	Forward	TGGAGCATGTGGTTTAATTCGA	160	Reference[17]
	Reverse	TGCGGGACTTAACCCAACA		
gtfB of *S. mutans*	Forward	AGCCATGCGCAATCACAGGTT	415	Reference[10]
	Reverse	CGCAACGCGAACATCTTGATCAG		
gtfI of *S. sobrinus*	Forward	GAAACCAACCCAACTTTAGCTTGGAT	319	Reference[18]
	Reverse	ATGGAGTGATTTTCCATCGGTACTTG		

Table 2. Biochemical characteristics of cultures grown from five dental plaque samples

Sample	Gram Staining	Catalase	Urease	VP	Fermentation of Glucose
1	Cocci +	-	-	+	+
2	Cocci +	-	-	+	+
3	Cocci +	-	-	+	+
4	Cocci +	-	-	+	+
5	Cocci +	-	-	+	+

Table 3. Cycle threshold (Ct) values obtained for 16S rRNA, *S. mutans gtfB* and *S. sobrinus gtfI* gene amplification.

Dental plaque sample	Cycle threshold (Ct) Values				
	Target gene: 16S rRNA	Target gene: *gtfB* (S. *mutans)*		Target gene: *gtfI* (S. *sobrinus)*	
	Spin column purified DNA	Spin column purified DNA	Magnetic beads purified DNA	Spin column purified DNA	Magnetic beads purified DNA
1	20.29	33.02	30.30	39.33	25.33
2	20.21	33.80	31.18	36.57	31.12
3	20.23	34.31	30.01	38.64	32.20
4	20.52	34.90	31.00	35.44	34.11
5	20.20	31	30.55	36.38	33.11

of DNA from the A260/A280 ratio. Agarose gel electrophoresis of the isolated DNA samples confirmed the DNA quality.

Semi-quantitative Real-time Polymerase chain reaction

The purified genomic DNA samples from dental plaques had to be confirmed with 16S rRNA universal primers whether it is of bacterial origin. Using gene specific primers or 16S rRNA universal primers, the reaction mixture in a total volume of 20il contained 2il of nuclease free water, 10il SYBR® green qPCR master mix, 1.5il each of forward and reverse gene specific primers or 1.5il each of forward and reverse 16S rRNA universal primer, and 5il of bacterial genomic DNA. The C1000™ thermocycler with the CFX 96 Real-

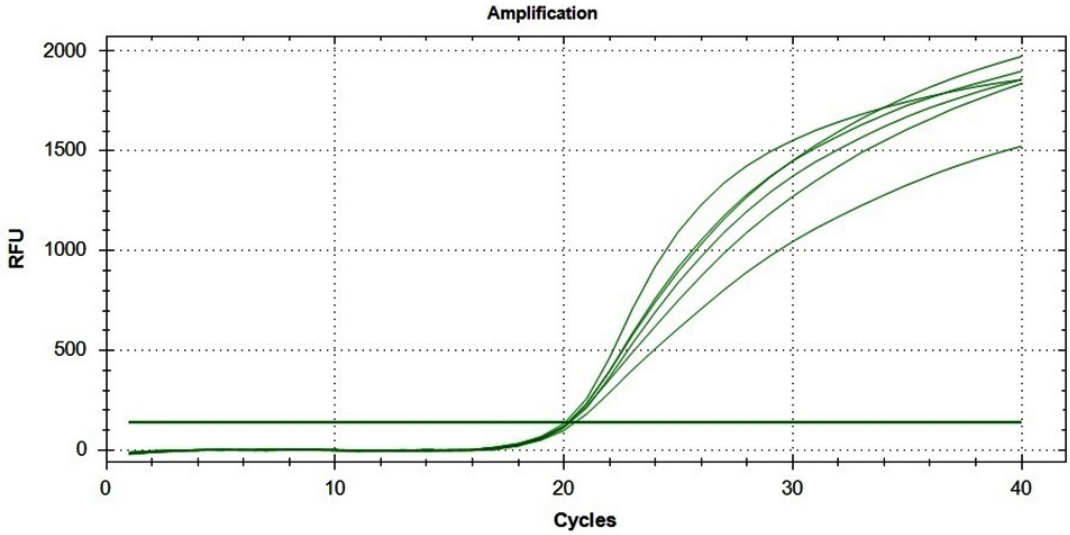

Fig. 1. SYBR Green based qRT-PCR. Amplification curve [Relative Fluorescence Unit (RFU) versus Cycle Threshold (Ct value)] represents Ct values for five dental plaque samples for 16S rRNA gene

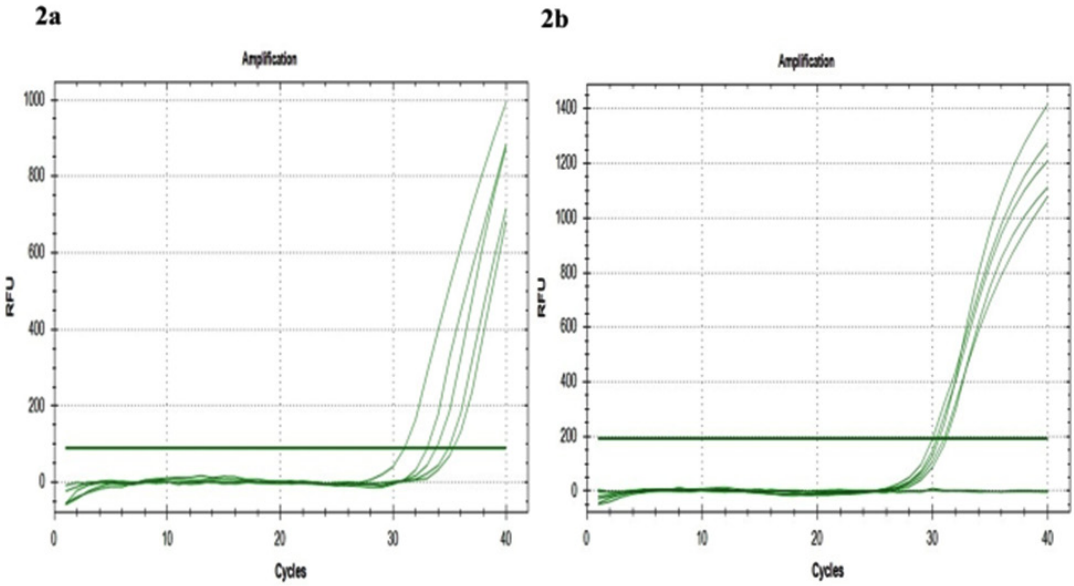

Fig. 2. SYBR Green based qRT-PCR. Amplification curve [Relative Fluorescence Unit (RFU) versus Cycle Threshold (Ct value)] represents Ct values for five dental plaque samples for (2a) *S. mutans gtfB* gene using spin column purified DNA and (2b) *S. mutans gtfB* gene using magnetic beads purified DNA

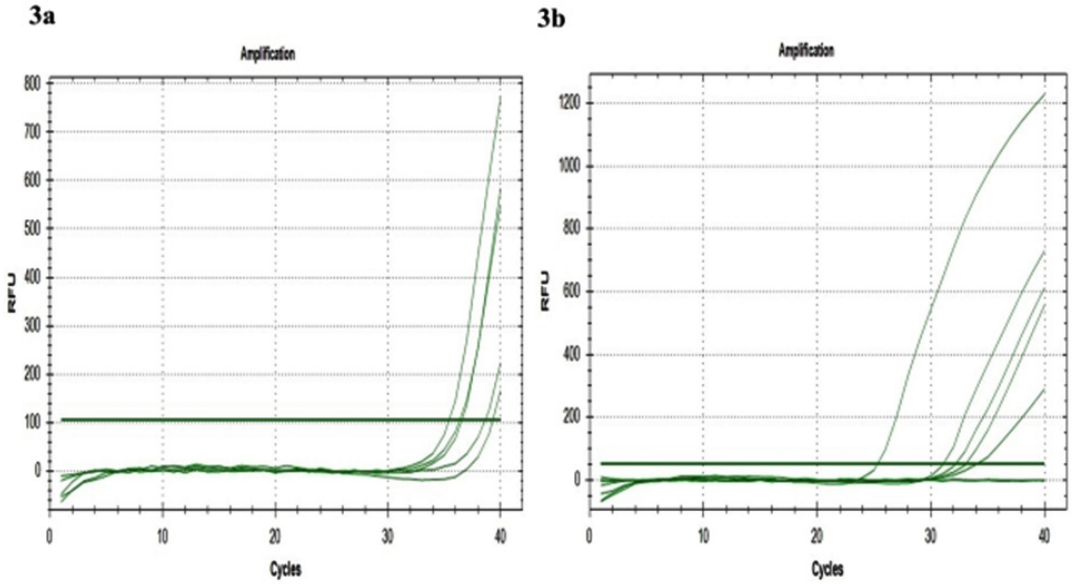

Fig. 3. SYBR Green based qRT-PCR. Amplification curve [Relative Fluorescence Unit (RFU) versus Cycle Threshold (Ct value)] represents Ct values for five dental plaque samples for (3a) *S. sobrinus gtfI* gene using spin column purified DNA and (3b) *S. sobrinus gtfI* gene using magnetic beads purified DNA.

time System (Biorad, USA) was set for 40 cycles. Each cycle consisted of an enzyme activation step at 95°C for 10 minutes followed by denaturation at 95°C for 15 seconds, annealing and extension at 60°C for 1 minute. The amplification curve was obtained for each gene specific primer or 16S rRNA universal primer at the end of each PCR reaction. A negative control (water blank) was included while performing the PCR. Sterile deionized water was used as a negative control to detect any potential contamination. The sequences of the 16S rRNA universal primer, *S. mutans gtfB* and *S. sobrinus gtfI* primers are listed in Table No. 1

Culture conditions

The dental plaque samples collected in the Biopro oral microbiome transport media were streaked on sterile Nutrient agar plates. The plates were incubated overnight at 37!. The microbial colonies grown were examined by Gram's staining & subjected to various biochemical tests.

Biochemical tests - Catalase test

A loop-full of all the five overnight grown cultures were transferred on five clean, well labelled microscopic glass slides in a sterile environment. After the addition of a drop of 3% Hydrogen peroxide on each glass slide, the slides were observed for the formation of gas bubbles[19].

Urease test

The tubes containing Urea agar slants were inoculated with a loopful of overnight grown culture and the tubes were incubated at 37! for 48 hours and observed for colour change[20].

DISCUSSION

The most widespread non-communicable disease in the world affecting both adults and children is Dental caries, which causes tooth decay or dental cavities. Dental plaque, which adheres to the tooth surface as a soft gelatinous mass comprises of bacteria embedded in an organic matrix. Streptococcus species are among the first to colonize a tooth surface, thereby initiating the formation of dental plaque, which eventually may cause caries[23]. *S. mutans* and *S. sobrinus*, the two members of Streptococcus species, are the most prevalent cariogenic bacteria found in humans and are considered the principal etiologic agents of dental caries disease[24]. The identification of anaerobic cariogenic bacteria by the standard traditional culture method is not always sensitive enough and requires special technical skill while isolating fastidious anaerobic bacteria. The main goal of this study was to develop a culture-

independent diagnostic test to verify the presence of the microorganisms in dental plaques. Bacterial small subunit 16S rRNA gene is used to detect bacterial pathogens. The Semi-quantitative Real-Time PCR amplification of the 16S rRNA gene using 16S rRNA universal primers confirmed that the purified genomic DNA obtained from the dental plaque samples was indeed of bacterial origin.

Previous studies have revealed that RT-PCR detection is an effective method as compared to other biochemical tests to distinguish *S. mutans* from other oral Streptococci species[25]. RT-PCR method is quick, relatively simple, and low concentrations of template DNA is sufficient for the analysis. Previously, a comparative study was conducted to detect the presence of *S. mutans* in the saliva of children with that of adults and adolescents[25]. They concluded that the saliva samples harvested from children contained significantly lower *S. mutans* as compared to saliva samples from adults and adolescents. However, plaque samples collected from children were found to contain *S. mutans* noticeably more frequently than plaque samples collected from adolescents and adults[25].

In the present study, Semi-quantitative Real-time PCR analysis using species specific glucosyltransferase primers was performed in order to identify *S. mutans* and *S. sobrinus* species. All the five dental plaque samples were individually analyzed for the presence of the two targeted bacterial species. The purpose of this study was to only confirm the presence of the two predominant cariogenic Streptococcus species using Real-time PCR analysis. Approximately 10 – 20 nano grams of the template bacterial genomic DNA was sufficient in the reaction mixture for detecting the two major Streptococcus species using the cost effective qRT-PCR method. In order to identify the non culturable and other diverse bacterial species from the bacterial genomic DNA isolated from dental plaque samples access to a gene sequencer was essential. To assess the complete composition of the bacterial species present in a dental plaque sample high cost new molecular biology techniques like 16S rRNA gene next generation sequencing (NGS), whole-genome sequencing (WGS) or whole metagenome shotgun sequencing needs to be performed.

CONCLUSION

Firstly, Genomic DNA was purified from the dental plaque samples using the Biopro spin column and magnetic beads kit. DNA purification using spin columns produced better quantity and quality of DNA than using magnetic beads. The purified Genomic DNA was confirmed whether it is of bacterial origin by Semi-quantitative Real-time PCR detection of the bacterial 16S rRNA gene using the universal 16S rRNA primers. Semi-quantitative real-time PCR was used to detect the presence of both the cariogenic Streptococcus species (*S. mutans* and *S. sobrinus*) using the *S. mutans gtfB* and *S. sobrinus gtfI* gene specific primers and SYBR Green fluorescent dye for real-time detection. Real-time PCR is a feasible non-invasive diagnostic method for microbial community analysis, helps in the early detection of dental caries disease, and the results can be obtained within 2 hours.

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Conflict of interest

There is no conflict of interest.

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