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Quantitative detection of cariogenic *Streptococcus mutans* from saliva using TaqMan assay by Real-Time PCR: A case study in Allahabad

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Abstract

Mutans streptococci have been implicated as cariogenic bacteria in dental caries because they can produce high levels of dental caries-causing lactic acid and extracellular polysaccharide. The aim of this study was to isolate and characterize the mutans streptococci from the saliva obtained from the peoples of Allahabad. The specifically designed TaqMan probe based Geno-Sen's SM Kit for quantitative detection of *Streptococcus mutans* using Real-Time PCR model Rotor Gene 6000 (Corbett Research, Australia) on the basis of *gtfB* gene. The specific master mix contains reagents and enzymes for the specific amplification of *S. mutans* and for the direct detection of the specific amplificon in fluorescence channel cycling Green (FAM) of the Rotor Gene 6000 and the reference gene on cycling Yellow (Joe) for external positive standards of *S. mutans* MTCC-890. In this study all previously biochemically identified cariogenic clinical isolates were used for TaqMan assay. Out of 10 isolates of *Streptococcus* sp. 6 isolates namely as MS1; MS9; MS36; MS45; MS54 and MS72 were amplified and found 1079324; 93182; 145439; 13696; 25011 and 3501577 copies/ml. Standard *S. mutans* MTCC-890 has also amplified and showed 3524945 copies/ml. Using this assay, the numbers of cariogenic *S. mutans* bacteria in saliva and dental plaque was observed. Present investigation revealed that the TaqMan assay is accurate and useful for the absolute and relative quantification of cariogenic bacteria from oral specimens.

Keywords: Biochemical; Geno-Sen's SM; Quantitative; Real-Time PCR; TaqMan; *Streptococcus mutans*

INTRODUCTION

Dental caries is one of the most common infectious diseases afflicting humans (1). Although 200 to 300 bacterial species have been found in saliva, *Streptococcus mutans* has been considered as a potent caries causing bacteria (2). Saliva is a complex mixture of several components (3). Whole saliva (oral fluid) is formed primarily from salivary gland secretions, but also contains gingival fluid, desquamated epithelial cells, bacteria, leucocytes, and possibly food residues, blood and viruses (4). Saliva is essential for maintenance of healthy oral tissues; it coats the oral mucosa and protects against irritation, forms an ion reservoir for tooth remineralization, functions as a buffer, aids in swallowing, exerts antimicrobial action, participates in pellicle formation and enzymic digestion of starch with amylase, and also participates in taste sensation by acting as a solvent (3).

But the bacterial content of saliva is estimated to approach 109 bacteria per ml [5]. Saliva helps to control invasion of the mouth by microorganisms, and lack of saliva results in increased numbers of bacteria in the mouth. Saliva can act as a selective medium for bacterial growth, but continuously repeated swallowing results in clearing of bacteria (5). Salivary mutans streptococcal counts rarely exceed 10^7 CFU per ml A highly significant correlation has been demonstrated between the salivary numbers of mutans streptococci and their prevalence in the dentition, both in terms of the number of tooth surfaces colonized and the level of infection of tooth surfaces (6).

Various methods have been used to detect and identify the oral streptococcal species including biochemical tests (7), immunological tests (8), DNA probes (9), Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (10), PCR (11) and 16S rDNA sequencing comparison methods (12). Among them, the PCR method is currently being applied to the detection of putative pathogens and the identification of human cariogenic bacteria because it is rapid, sensitive, and simple. The target genes for the PCR are often related to virulence factors such as dextranase gene (dex) (11) or 16S ribosomal RNA gene (16S rDNA) (13,14). Most of the PCR-based diagnosis systems reported are qualitative analysis and are therefore unsuitable for accurate evaluation of caries susceptibility or caries activity. Quantitative analysis is essential for monitoring the cell number and/or ratio of cariogenic bacteria in oral specimens, such as dental plaque and saliva.

A real-time PCR assay with the TaqMan system based on the 5'-3' exonuclease activity of *Taq* polymerase has been developed for the quantitative detection of DNA copy number (15). Briefly, an oligonucleotide probe with a reporter fluorescent dye attached to its

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5' end and a quencher dye attached to its 3' end is designed to hybridize to the target gene. During PCR amplification, the quencher dye of the probe is cleaved by the 5' nuclease activity of *Taq* polymerase, resulting in the accumulation of reporter fluorescence. The release of the fluorescent dye during amplification allows for the rapid detection and quantification of DNA (16).

MATERIALS AND METHODS

Saliva Sample Collection

Saliva from decayed tooth portions was collected in sterilized test tube with the help of sterilized cotton swabs and transferred into transport medium and isolation part was done at Biological Product Laboratory, Department of Botany, University of Allahabad, Allahabad -211 002.

Isolation of bacterial isolates

Vortexed saliva sample and transferred 5 µl in to the Hewitt Todd broth tubes with the help of micropipette. Samples were incubated aerobically as well as anaerobically in candle extinction jar (Pressure of 1% CO_2) at 37ºC for 24 hours. Both aerobic and anaerobic incubated Hewitt Todd broth showed turbidity and it was clear that the dental caries causing organism were aerobic as well as facultatively anaerobic. Aerobically incubated samples were streaked on Mitis Salivarius agar plates (specific medium for *Streptococcus* sp.) and incubated at 37ºC for 48 hours under microaerophilic condition (17). Bacterial colonies from Mitis Salivarius agar were collected for biochemical as well as Molecular identification.

Biochemical Identification

The biochemical tests were performed to identify the isolated organisms using Hi strep TM Identification kit KB005 from Hi-media and other biochemical tests such as Hemolysis in blood agar, Starch hydrolysis, Urease hydrolysis and Gelatin liquefaction with one reference strain of *Streptococcus mutans* MTCC-890.

Molecular Identification

As a representative microorganism of the oral microbiota, biochemically characterized 10 *Streptococcus* spp. isolates with one MTCC-890 strain of *Streptococcus mutans* were used in this study, mainly because of their active involvement in dental diseases.

Isolation of DNA form cariogenic isolates

AccuPrep® Genomic DNA Extraction Kit form Bioneer (USA) rapidly and conveniently extracts an average of 6µg of total DNA from 10^4-10^8 bacterial cultured cells. *AccuPrep*® Genomic DNA Extraction Kit employs glass fibers, fixed in column that specifically binds DNA in the presence of a chaotropic salt. Proteins and other contaminants were eliminated through a series of short wash and spin steps. Finally, genomic DNA was eluted by a low salt solution. This process does not require phenol/chloroform extraction, alcohols precipitation, or other burden some steps.

Real Time PCR assay

It was the validation and quantitative detection of *S. mutans* by using a TaqMan assay. In this study previously biochemically identified cariogenic clinical isolates were used. Genomic DNA was isolated and purified using *AccuPrep*® DNA isolation kit (Bioneer USA) in accordance with the manufacturer's instructions for gram-positive bacteria. Procedure of DNA isolation was given previously. Greisen *et al.* (18) and Yoshida *et al.* (19) were designed universal primers and a probe for a board range of bacteria. The *S. mutans* gene specific primers and probes were designed from the *gtfB* gene (20, 21). The specificities of the primers and probes were initially confirmed by BLAST with the National Center for Biotechnology Information. The robust assay exploits the so-called TaqMan principle. During PCR, forward and reverse primers hybridize to a specific sequence product. A TaqMan probe, which is contained in the same reaction mixture and which consists of an oligonucleotide labeled with a 5'-reporter dye and a downstream, 3'-quencher dye, hybridizes to a target sequence within the PCR products. A Taq polymerase which possesses 5'-3' exonuclease activity cleaves the probe. The reporter dye and quencher dye separated upon cleavage, resulting in an increase in fluorescence for the reporter. Thus, the increase in fluorescence is directly proportional to the target amplification during PCR.

Amplification of dental caries causing bacterial isolates by Real Time PCR

Each PCR reaction consisted of 10 mM Tris-HCl buffer (pH 8.3), 1.5mM $MgCl_2$, 50mM KCl, 200µM each of dATP, dTTP, dGTP, and dCTP or use 2.5X Universal Hot Start PCR Premix Cat. No. 110145 (Professional Biotech India Ltd., New Delhi). 1µM primers (GtfB-F and GtfB-R), 1U *Taq* DNA polymerase and 2µl of supernatant from the DNA extraction (Table 1 and 2).

Table 1 Dilution Protocol for PCR with 2.5 X Hot Start PCR Premix

Component	50 µl reaction Volume		25 µl reaction volume	
	Volume	Final Conc.	Volume	Final Conc.
2.5 X Hot Start PCR Premix	20 µl	1 X	10 µl	1 X
Forward primer	Variable	0.1-1 µM	Variable	0.1-1 µM
Reverse Primer	Variable	0.1-1 µM	Variable	0.1-1 µM
Template DNA	Variable	10pg-1 µg	Variable	10pg-1 µg
Sterile Deionized water	Up to 50 µl	----	Up to25 µl	----

-- It was advisable to use 20 µl of extracted template DNA in case low yield was expected for 50 µl reaction volumes. In case of High yields the volumes were adjusted as per the yield of DNA.

Table 2 Oligonucleotide primers and probes on the basis of gtfB gene

Designation	Sequence* Accession number are AE015003 for the *S.mutans* gtfB gene	Amplicon size (bp)	Target
Primers			
Smut-F	5' –ACTACACTTTCGGGTGGCTTGG-3'	517	gtfB
Smut-R	5' –CAGTATAAGCGCCAGTTTCATC-3'		
Fluorescent Probes			
Smut 3423T	5'–FAMTGGAAATGACGGTCGCCGTTATG AA- TAMRA-3'		gtfB

Denaturation at 95°C for 30 seconds, followed by annealing at 59ºC for 30 seconds, and extension at 72ºC for one minute. The amplification was repeated for 25 cycles using a machine Rotor gene 6600 Corbett Research Australia.

Geno-Sen's SM kit

The Geno-Sen's SM kit was used for detection and quantification of *Streptococcus mutans* using RT-PCR in the Rotor Gene 6600 (Corbett Research, Australia). It contained Premix 12µl; MgCl2 2.5µl; IC gene 0.5µl and 10µl bacterial DNA to make total volume 25µl for amplification of one reaction. The specific master mix contains reagents and enzymes for the specific amplification of *S. mutans* and for the direct detection of the specific amplificon in fluorescence channel cycling Green (FAM) of the Rotor Gene 6600 and the reference gene on cycling Yellow (Joe) for external positive standards of *S. mutans* MTCC-890 (Table 1).

RT-PCR Inhibition control

Inhibition control gene was allows to determine and controlled possible RT-PCR inhibition. The inhibition gene reagents were in built in the premix provided and needed not to be run separately. It was required to add internal control gene in the reaction mixture, which has been provided as IC gene. 1 µl/ reaction of IC gene was added.

Quantitation of bacterial gene copies

The quantitation standards provided in the Kit (Geno-Sen's SM) were treated in the same way as extracted samples and the same value was used *i.e.* (25µl) instead of the sample. To generate a standard curve in the Rotor Gene 6000, all 5 standards should be used as defined in the menu window edit samples of the Rotor Gene software. The same should also be defined as standards with the specified concentrations. The standard curves generated as above can also be use for quantitation in subsequent runs, provided that at least one standard is used in the current run. For this purpose, the previously generated standard curve needs to be imported (Fig. 2 and 3). However, this quantitation method may lead to deviations in the results due to variability between different PCR runs and due to varying reaction efficiencies.

$$\text{Results (Copies/ml)} = \frac{\text{Results (Copies/µl) x Elution volume (µl)}}{\text{Sample volume (ml)}}$$

RESULTS

Isolation of cariogenic pathogens

In the present study, out of 87 caries causing clinical isolates collected from saliva of different peoples in Allahabad. 10 isolates were randomly selected for identification by morphological and biochemical characterization compared with MTCC 890 standard strains and confirmation of isolates by using Real-Time PCR assay.

Biochemical characteristics of bacterial isolates

Biochemical characterization of caries causing bacterial isolates with the help of certain biochemical parameters such as Starch hydrolysis, Urease hydrolysis etc. and Hi Strep TM Kit KB005 from Hi-media. The bacterial isolates, MS1, MS9, MS18, MS36, MS45, MS54, MS63 and MS72, which were isolated from Mitis salivarius agar showed haemolysis in blood agar and other biochemical test similarly like *Streptococcus mutans* MTCC 890 (Table 3).

The identification of mutans streptococci was based on distinctive colonial morphology on selective and non-selective agar, Gram staining, distinctive cell shape on light microscopy, specific growth characteristics, and sugar fermentation and enzymatic patterns. The identification was based on information in Bergey's Manual of Determinative Bacteriology (9th ed., 1994) and Hi Strep TM Kit KB 005 was used for the identification of *Streptococcus mutans*. Further, on starch hydrolysis, all the identified *S.mutans* showed amylase activity (Table 3).

Validation and quantitative identification by RT-PCR

This study has done for validation and quantification of *S. mutans* isolates with the help of modern molecular tool *i.e.* Real-Time PCR. All isolates of *Streptococcus mutans*, those were identified previously also showed amplification with using TaqMan assay. Out of 10 isolates of *Streptococcus* sp. 6 isolates namely as MS1; MS9; MS36; MS45; MS54 and MS72 were amplified and found 1079324; 93182; 145439; 13696; 25011 and 3501577 copies/ml. Standard *S. mutans* MTCC-890 was also amplified and showed 3524945 copies/ml. The quantification results are given in Table 4.

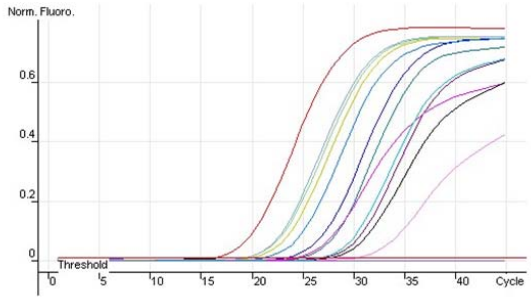

Figure 1 Quantitation data for Cycling A. Green

Amplification plots of chromosomal DNA from lysed cells. Serial dilutions of chromosomal DNA were from *S. mutans.* The log-transformed relative fluorescence [ΔRn(log)] was monitored as the increase in reporter dye intensity relative to the passive internal reference dye. The threshold fluorescence, or level at which the threshold cycle was determined, is shown

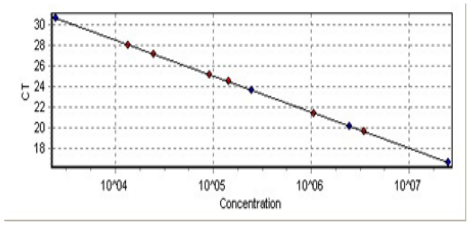

Figure 2 Standard curves were generated from the amplification plots in the small panels (Correlation coefficient = 0.996 for *S. mutans*). Ct is the cycle number at which the threshold fluorescence was reached

Table 3 Morphological and biochemical characteristics of dental caries causing *Streptococcus* sp.

S. No.	Characteristics	Dental caries causing bacterial isolates										MTCC 890
		MS 1	MS 9	MS 18	MS 27	MS 36	MS 45	MS 54	MS 63	MS 72	MS 87	
1.	Grams reaction	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
2.	Shape	cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci
3.	Growth at 37°C	good	good	good	good	good	good	good	good	good	good	good
4.	Hemolysis on Blood Agar	γ	γ	γ	β	γ	γ	γ	γ	γ	γ	γ
5.	Starch Hydrolysis	+	+	+	+	+	+	+	+	+	+	+
6.	Simmon Citrate Utilization	-	-	-	+	-	-	-	-	-	+	-
7.	Urease Hydrolysis	-	-	-	+	-	-	-	-	-	+	-
8.	Gelatin liquefaction	-	-	-	+	-	-	-	-	-	+	-
9.	Voges proskauer's	+	+	+	-	+	+	+	-	+	-	+
10.	Esculin hydrolysis	+	+	-	-	+	+	+	-	+	-	+
11.	PYR.	-	-	+	+	-	-	-	+	-	+	-
12.	ONPG	-	-	-	-	-	-	-	-	-	-	-
13.	Arginine utilization test	-	-	+	+	-	-	-	-	-	+	-
14.	Glucose	+	+	+	+	+	+	+	+	+	+	+
15.	Ribose	+	+	+	+	+	+	+	+	+	+	+
16.	Arabinose	±	-	-	-	-	±	-	-	-	-	±
17.	Sucrose	+	+	+	+	+	+	+	+	+	+	+
18.	Sorbital	+	+	+	-	+	+	+	+	+	-	+
19.	Mannitol	+	+	+	-	+	+	+	+	+	-	+
20.	Raffinose	+	+	+	-	+	+	+	+	+	-	+
Identification		*S. mutans*	*S. mutans*	*S. mutans*		*S. mutans*	*S. mutans*	*S. mutans*	*S. mutans*	*S. mutans*		*S. mutans*

Table 4. Amplified DNA copies from Clinical Isolates of *S. mutans* (copies/ml)

No.	Colour	Name	Type	Ct	Given Conc (copies/ml)	Calc Conc (copies/ml)	% Var
1		S1 *S. mutans* with Geno-Sen's RT-PCR quantitation	Standard	16.64	25000000	25191406	0.8%
2		S2	Standard	20.13	2500000	2515345	0.6%
3		S3	Standard	23.65	250000	245648	1.7%
4		S4	Standard	27.13	25000	24659	1.4%
5		S5	Standard	30.57	2500	2544	1.8%
6		MS 1	Unknown	21.41		1079324	
7		MS 9	Unknown	25.12		93182	
8		MS 18	Unknown				
9		MS 27	Unknown				

No.	Colour	Name	Type	Ct	Given Conc (copies/ml)	Calc Conc (copies/ml)	% Var
10		MS 36	Unknown	24.44		145439	
11		MS 45	Unknown	28.02		13696	
12		MS 54	Unknown	27.11		25011	
13		MS 63	Unknown				
14		MS 72	Unknown	19.63		3501577	
15		MS 87	Unknown				
16		*S. mutans* MTCC-890	Unknown	19.62		3524945	
17		NC	NTC				

The report was generated using Rotor-Gene Real-Time Analysis Software 6.0 (Build 33) (C)Corbett Research 2004 (R)All Rights Reserved

It is concluded that clinical isolate MS72 was closely related to standard *Streptococcus mutans* MTCC-890 strains on behalf of numbers of amplified DNA copies/ml. It also proves that biochemically identified *Streptococcus* sp. Clinical isolates MS18 and MS63 are not belonging to *Streptococcus mutans* because it did not amplified during Taqman assay in Real-Time PCR (Fig. 1 and 2).

Oligonucleotide primers and probes were designated to amplify DNA at 517bp- G*tf*B sequence of *S. mutans* (Table 2).

DISCUSSION

On MS agar, *S. mutans* colonies were small, raised, irregularly margined and adherent, while *S. sobrinus* colonies are surrounded by a zooglea with a gelatinous consistency (22). On blood agar incubated anaerobically for two days, *S. mutans* colonies were white or gray, circular or irregular, 0.5-1.0 mm in diameter, sometimes tending to adhere to the surface of the agar (23). For primary isolation of mutans streptococci, the most frequently used medium is mitis salivarius (MS) agar (17). However, in the present study Mitis salivarius agar was also taken for the culture identification of *Streptococcus mutans.*

The identification of mutans streptococci was based on distinctive colonial morphology on selective and non-selective agar, Gram staining, distinctive cell shape on light microscopy, specific growth characteristics, and sugar fermentation and enzymatic patterns. The identification was based on information in Bergey's Manual of Detrerminative Bacteriology (9th ed., 1994) and Corredoira *et al.,* (24). However, in the present study Hi Strep TM Kit KB 005 was used for the identification of Streptococci on species level.

S. mutans has been reported to have extracellular amylase activity, on the basis of hydrolysis of starch in agar plates. The protein encoded by *amy* does not contain a signal peptide typical of secreted proteins and appears to be located predominantly intracellularly. However, it was responsible for the clearing of starch observed around colonies, because the mutant in the *amy* gene had been insertion ally inactivated was unable to hydrolyze starch in agar plates. The mechanism by which some of the amylase activity escapes the cell is unknown, *S. mutans* required exogenous α-amylase in order to hydrolyze starch (25), addition of salivary α-amylase (10U/ml; Sigma) enabled *S. mutans* to produce acid from starch (data not shown). *S. mutans* can thus utilize limit dextrins from starch digestion, which can be taken up by the multiple sugar metabolism transport system (26). However, in the present study, all the identified *S. mutans* strain from the saliva was also hydrolysis of starch showed amylase activity.

In this study, out of 10 isolates of Streptococcus spp., 6 spp namely MS1; MS9; MS36; MS45; MS54 and MS72 were amplified and found 1079324; 93182; 145439; 13696; 25011 and 3501577 copies/ml. Standard *S. mutans* MTCC-890 was also amplified and showed 3524945 copies/ml. The quantification results are given in Table 4. The numbers of these organisms in each saliva sample varied by several orders of magnitude, and our results for saliva samples were consistent with a previous report (27). In addition, the relative amounts of these organisms in oral specimens were calculated by the comparative Ct method (19), with a simplification. Briefly, the results, expressed as the fold deference (*N*) between the number of target gene copies and the number of 16S rRNA gene copies were determined.

According to Oho *et al.* (20) Oligonucleotide primers and probes were designated to amplify DNA at 517bp of the G*tf*B sequence of *S. mutans* (Table 2). The denaturation at 95C for 30s followed by annealing at 59°C for 30s and extension at 72°C for 1 min. This amplification was repeated for 30 cycles. PCR products were subjected to electrophoresis on 1.5% agarose gel and stained with ethidium bromide. With the primers used, *S. mutans* produced single DNA fragments.

S. mutans have a significantly higher caries incidence and incremental increase than those with *S. mutans* alone. Our results suggested that the PCR method employed was suitable for investigating the intra-oral distribution of *S. sobrinus* as well as *S. mutans*, as the 16S rDNA primers used confirmed the presence of bacteria in all plaque samples. This tool provides a more sensitive means of detection of cariogenic bacterial species, as compared to conventional culture techniques (28, 11). Therefore, this study demonstrated that Real-Time PCR is a reliable, accurate and quantitative method for the detection of *S. mutans* in epidemiological studies. It was also observed that children presenting *Streptococcus mutans has* potential to develop caries.

CONCLUSION

This investigation revealed that the TaqMan based Real-Time PCR Kit Geno-Sen's SM was accurate and useful for the absolute and relative quantification of cariogenic bacteria from oral specimens. Recently, dental plaque has been considered an oral biofilm, and monitoring the absolute or relative amount of cariogenic bacteria in oral biofilm was essential. Moreover, the TaqMan PCR-based quantification system is advantageous, since cell numbers can be

monitored in the biofilm as it is. This assay system will be useful for clarifying how these bacteria behave in oral biofilm formation and will contribute to the development of biofilm research.

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REFERENCES

[1] Koga, T., Oho, T., Shimazaki, Y., Nakano, Y. 2002. Immunization against dental caries. Vaccine; 20:2027-2044.

[2] Kuramitsu, H.K. 2001. Virulence properties of oral bacteria: impact of molecular biology. Curr. Issues Mol Biol., 3:35-36.

[3] Whelton, H. 1996. Introduction: the anatomy and physiology of the salivary glands. In: Saliva and oral health.eds. 2nd ed. British Dental Journal: London; pp.1-8.

[4] Dawes, C. 1996. Factors influencing salivary flow rate and composition. In: Saliva and oral health. Edgar WM, O'Mullane DM, eds. 2nd ed. British Dental Journal: London; pp. 27-41.

[5] Bowen, W.H. 1996. Salivary influences on the oral microflora. In: Saliva and oral health. Edgar WM, O'Mullane DM, eds. 2nd ed. British Dental Journal, London; pp. 95-103.

[6] Duchin, S., van, Houte, J. 1978. Colonization of teeth in humans by *Streptococcus mutans* as related to its concentration in saliva and host age. Infect Immun., 20:120-125.

[7] Beighton, D., Russell, R.R.B., Whiley, R.A. 1991. A simple biochemical scheme for the differentiation of *Streptococcus mutans* and *Streptococcus sobrinus.* Caries Res 25: 174-178.

[8] de Soet, J.J., van Dalen, P.J., Russell, R.R., de Graaff, J. 1990. Identification of mutans streptococci with monoclonal antibodies. Antonie van Leeuwenhoek 58: 219-225.

[9] Cangelosi, G.A., Iversen, J.M., Zuo, Y., Oswald, T.K., Lamont, R.J. 1994. Oligonucleotide probes for mutans streptococci. Mol. Cell Probes; 8: 73-80.

[10] Igarashi, T., Ichikawa, K., Goto, N. 2001. Identification of mutans streptococcal species by the PCR products of the *dex* genes. J Microbiol Methods; 46: 99-105.

[11] Igarashi, T., Yamamoto, A., Goto, N. 1996. Direct detection of Streptococcus mutans in human dental plaque by polymerase chain reaction. Oral Microbiol Immunol; 11:294-298.

[12] Kawamura, Y., Hou, X.G., Sultana, F., Liu, S., Yamamoto, H., Ezaki, T. 1995. Determination of 16S rRNA sequences of *Streptococcus mitis* and *Streptococcus gordonii* and phylogenetic relationships among members of the genus *Streptococcus*. Int J Syst Bacteriol; 45:406-408.

[13] Rupf, S., Merte, K., Eschrich, K., Stosser, L., Kneist, S. 2001. Peroxidase reaction as a parameter for discrimination of *Streptococcus mutans* and *Streptococcus sobrinus.* Caries Res; 35: 258-264.

[14] Choi, M.H., Yoo, S.Y., Kang, D.W., Lim, C.K., Kook, J.K. 2006. Nested PCR for the detection of *Streptococcus mutans*. Kor J Microbiol; 42: 19-25.

[15] Holland, P.M., Abramson, R.D., Watson, R., Gelfand, D.H. 1991. Detection of specific polymerase chain reaction product by utilizing the 5'–3'exonuclease activity of *Thermus aquaticus* DNA polymerase. Proc. Natl. Acad. Sci. USA; 88:7276-7280.

[16] Heid, C.A., Stevens, J., Livak, K.J. 1996. Williams PM. Real time quantitative PCR. Genome Res; 6:986-994.

[17] Gold, O.C., Jordan, H.V., Van Houte, J. 1973. A selective medium for *Streptococcus mutans*. Arch Oral Biol., 18:1357-1364.

[18] Greisen, K., Loeffelholz, M., Purohit, A., Leong, D. 1994. PCR primers and probes for the 16S rRNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid. J Clin Microbiol; 32:335-351.

[19] Yoshida, A., Suzuki, N., Nakano, Y., Oho, T., Kawada, M., Koga, T. 2003. Development of a 5'fluorogenic nuclease-based real-time PCR assay for quantitative detection of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*. J Clin Microbiol; 41:863-866.

[20] Oho, T., Yamashita, Y., Shimazaki, Y., Kushiyama, M., Koga, T. 2000. Simple and rapid detection of *Streptococcus mutans* and *Streptococcus sobrinus* in human saliva by polymerase chain reaction. Oral Microbiol Immunol; 15:258-262.

[21] Shiroza, T., Ueda, S., Kuramitsu, H.K. 1987. Sequence analysis of the gtfB gene from *Streptococcus mutans*. J Bacteriol; 169:4263-4270.

[22] Hamada, S., Slade, H.D. 1980. Biology, immunology, and cariogenicity of *Streptococcus mutans*. Microbiol Rev.; 44: 331-384.

[23] Hardie, J.M. 1986. Oral streptococci. In: Bergey's manual of systematic bacteriology. Volume 2.Sneath PHA, Mair NS, Sharpe ME, Holt JG, eds. Williams and Wilkins: Baltimore, Maryland; pp. 1059-1063.

[24] Corredoira, J., Alonso, M.P., Coira, A., Varela, J. 2008. Association between *Streptococcus infantarius* (Formerly S. bovis II/1) Bacteremia and Noncolonic Cancer. Journal of Clinical Microbiology; 46(4):1570.

[25] Clarkson, B.H., Krell, D., Wefel, J.S., Crall, J., Feagin, F.F. 1987. *In-vitro* caries-like lesion production by *Streptococcus mutans* and *Actinomyces viscosus* using sucrose and starch. J Dent Res; 66:795-798.

[26] Tao, L., Sutcliffe, I.C., Russell, R.R.B., Ferretti, J.J. 1993. Cloning and expression of the multiple sugar metabolism (*msm*) operon of *Streptococcus mutans* in heterologous streptococcal hosts. Infect Immun; 61:1121-1125.

[27] Yano, A., Kaneko, N., Ida, H., Yamaguchi, T., Hanada, N. 2002. Real-time PCR for quantification of *Streptococcus mutans*. FEMS Microbiol Lett.; 217:23-30.

[28] Ono, T., Hirota, K., Nemoto, K., Fernandez, E.J., Ota, F., Fukui, K. 1994. Detection of *Streptococcus mutans* by PCR amplification of spaP gene. J Med Microbiol; 41, 231-235.