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Detection of *Streptococcus mutans* using Padlock Probe Based on Rolling Circle Amplification (RCA)

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ABSTRACT

The aim of this study was to develop and evaluate a padlock probe based on the Rolling Circle Amplification (RCA), which targeted to 16S-23S rDNA region of S. mutans*. The specificity of developed padlock probe was tested for DNA within a panel strains, including* S. mutans *isolated from the saliva and reference strains of the genus* Streptococcus*, as well as total DNA samples of biofilm and saliva. The results were positive either for DNA samples of* S. mutans *or DNA samples recovered from the biofilm and saliva revealing the specificity of designed padlock probe. The padlock probe based on the RCA was proved to be an effective, reproducible method for* S. mutans *detection and demonstrated the possibility of a rapid detection and accurate identification of* S. mutans *infection.*

Key words: Padlock probe, RCA, *Streptococcus mutans*

INTRODUCTION

Oral diseases related to dental biofilms still afflict the majority of the worldwide population. Among them, dental caries is the single most prevalent and costly oral infectious disease (Marsh 2003; Dye et al. 2007) resulted from the interaction of a specific bacterium with the constituents of the diet within a dental biofilm (Koo et al. 2010; Hsu et al. 2010) and host endogenous factors. Among hundreds of bacterial species in the human oral cavity, *Streptococcus mutans* plays a key role on the development of virulent biofilms of dental caries (Beighton 2005). However, the classical microbiological cultures method limits the studies about the identification of specific populations of *S. mutans*, due to over 100 recognized species in the genus *Streptococcus* (Nobbs et al. 2009).

In conventional studies, the streptococci classification was based on the Lancefield scheme, which groups streptococcal strains according to the carbohydrate composition of cell wall antigens (Lancefield 1933). Such antigens, known as group-specific antigens or C substances, are either polysaccharides (as in groups A, B, C, E, F, and G), teichoic acids (as in groups D and N), or lipoteichoic acid (as in group H) (Rosan 1973). This approach has been successful in some

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groups, but its widespread application is hindered by the fact of a group-specific antigens for other species, which may be absent or shared between distinct taxa.

The streptococci may be organized into six groups based on 16S rRNA gene sequences: Mitis, Anginosus, Pyogenic, Bovis, Salivarius and Mutans. Mutans in conjunction with a range of bacteria colonize the oral cavities of humans (*S. mutans* and *S. sobrinus*), macaques (*S. downei*), rats (*S. ratti*) and hamsters (*S. criceti*), which are all associated to the development of dental caries (Nobbs et al. 2009). Clinical laboratories use serological grouping by Lancefield, haemolytic reactions and phenotypic tests for the identification of various *Streptococcus* isolates. However, these Lancefield groups are not species-specific and haemolytic activity differs within the species and depends on incubation procedures. Strains within a given species may differ for a common trait and even the same strain may exhibit biochemical variability (Lal et al. 2011). Thus, genetic methods have been used to differentiate the isolates of *S. mutans* and *S. sobrinus*, the oral *Streptococcus* associated with dental plaque. PCR is currently being applied in a wide range of diagnosis and research involving the species of the genus *Streptococcus* (Al-Ahmad et al. 2006) due to its high specificity and sensibility.

A number of DNA-based probes and primers have been developed (Chen et al. 2004). Many of the specific probes or primers were targeted to specific genes associated with virulence in *S. mutans*, such as glucosyltransferases (Oho et al. 2000; Yano et al. 2002), fructosyltransferases (Smorawinska and Kuramitsu 1992), dextranase (Igarashi et al. 1996), glucan-binding protein B (Smith et al. 2003) or cell surface protein (Lee and Boran 2003). Several other sets of primers for PCR were designed to amplify the specific regions of the 16S rRNA genes of *S. mutans* (Wang et al. 2002; Yano et al. 2002; Yoshida et al. 2003; Hoshino et al. 2004). All of them have been developed on the basis of cultured material and require a fully equipped molecular laboratory. However, there still is a need for a rapid and simple technique, which is able to deliver an unambiguous identification within a single day.

In 1994, Nilson et al. discovered the use of a padlock probe to circularize oligonucleotides that were useful to the detection of nucleic acids specific for application in Rolling Circle Amplification (RCA). This padlock probe is a circularizable oligonucleotide consisting of two segments complementary to the 3' and 5' ends of the target and a linker sequence. When the 3' and 5' terminal regions of the oligonucleotide probes are juxtaposed to the sequence of interest, the probe ends can be joined by a DNA ligase to form a circular DNA molecule that can be amplified by RCA. Since then, this technique has been used for diagnosis and research, such as subtyping of *Streptococcus agalactiae*, serotype III (Tong et al. 2007); molecular identification of *Penicillium marneffei* (Sun et al. 2011), and for rapid molecular identification of black yeast like fungal pathogens (Najafzadeh et al. 2011). It is generally believed that RCA is a practicable option to discriminate the closely related species or single nucleic acid difference within the species (Sun et al. 2011). Therefore, this study aimed to develop species-specific padlock probes used in combination with rolling circle amplification for the *S. mutans* detection.

MATERIALS AND METHODS

Strains and specimens

Sixteen *S. mutans* strains isolated from the saliva from the individuals with cavity caries by sequence analysis of the 16S-23S ribosomal DNA spacer region (Chen et al. 2004) and three reference strains (Table 1) were used in this study. In addition, five samples of saliva and five samples of dental biofilm were tested.

DNA extraction and IGS region amplification

All *Streptococcus* isolates were cultivated in brain heart infusion (BHI) broth and incubated at 37ºC for 24 h. The obtained cultures had 1 x 10^8 cells/mL and were centrifuged at 49,000 g for 2 min. The precipitate was transferred to a mixture of powder silica and celite in CTAB buffer, and the DNA was extracted according to the methodology described by Moreira et al. (2010). The DNA samples of saliva and dental biofilm were obtained by using the same proceedings. The 16S-23S intergenic region was amplified from *S. mutans* isolates for total DNA from saliva DNA total or biofilm DNA total, using primers 13BF (5' GTGAATACGTTCCCGGGCCT 3') and 6R (5' GGGTTYCCCCRTTCRGAAAT 3') (Chen et al. 2004).

Table 1 - Sampling data of isolates used in this study.

Id. Name	Culture collection	Genbank Accession number	Source	Geography
Streptococcus mutans	LB. SM1	HE962158	Saliva	Brazil
	LB. SM2	HE962159	Saliva	Brazil
	LB. SM3	HE962160	Saliva	Brazil
	LB. SM4	HE962161	Saliva	Brazil
	LB. SM5	HE962162	Saliva	Brazil
	LB. SM6	HE962163	Saliva	Brazil
	LB. SM7	HE962164	Saliva	Brazil
	LB. SM8	HE962165	Saliva	Brazil
	LB. SM9	HE962166	Saliva	Brazil
	LB. SM10	HE962167	Saliva	Brazil
	LB. SM11	HE962168	Saliva	Brazil
	LB. SM12	HE962169	Saliva	Brazil
	LB. SM13	HE962170	Saliva	Brazil
	LB. SM14	HE962171	Saliva	Brazil
	LB. SM15	HE962172	Saliva	Brazil
	LB. SM16	HE962173	Saliva	Brazil
	LMICRO03-SM	KJ767147	Saliva	Brazil
	LMICRO04-SM	KJ767146	Dental Biofilm	Brazil
	LMICRO06-SM	KJ767148	Saliva	Brazil
	LMICRO11-SM	KJ767149	Dental Biofilm	Brazil
	LMICRO12-SM	KJ767140	Dental Biofilm	Brazil
	LMICRO14-SM	KJ767132	Dental Biofilm	Brazil
	LMICRO19-SM	KJ767133	Dental Biofilm	Brazil
	LMICRO20-SM	KJ767134	Saliva	Brazil
	LMICRO21-SM	KJ767141	Dental Biofilm	Brazil
	LMICRO23-SM	KJ767142	Dental Biofilm	Brazil
	LMICRO24-SM	KJ767135	Dental Biofilm	Brazil
	LMICRO26-SM	KJ767143	Biofilm	Brazil
	LMICRO27-SM	KJ767136	Dental Biofilm	Brazil
	LMICRO29-SM	KJ767144	Dental Biofilm	Brazil
	LMICRO33-SM	KJ767137	Dental Biofilm	Brazil
	LMICRO47-SM	KJ767138	Dental Biofilm	Brazil
	LMICRO59-SM	KJ767139	Dental Biofilm	Brazil
	LMICRO60-SM	KJ767131	Dental Biofilm	Brazil
	ATCC 25175	UO 2919	Decayed dentin	England
Streptococcus sobrinus	ATCC 33478	AY 188349	Dental Biofilm	Sweden
Streptococcus pyogenes	ATCC 13540	EF 613287	Oropharynx	USA

LB - Labmicro-Biomol of Federal University of Paraná State; HE - European Nucleotide Archive; ATCC – American Type Culture Collection

Padlock probe and primers

Padlock probe was design based on the intergenic region sequences 16S-23S of *Streptococcus* and *Enterococcus* genus in GenBank or Molecular Biology Laboratory data bank – Labmicro-Biomol, UFPR (Table 2).

The probe was designed as previously described by Wang et al. (2005). To ensure the efficiency of padlock probe binding, the padlock probes were designed with minimum secondary structure and with the Tm of the 5′ end probe binding arm close to or above the ligation temperature (60°C). To increase its discriminative specificity, the 3′ end binding arm was designed with a Tm 10°C–15°C below ligation temperature. The genetic linker region was designed to minimize any similarity to potentially cross-reacting sequences after BLAST search. The primers used to amplify the specific padlock probe signal during RCA were designed specifically to bind to the linker regions with a Tm of about 55°C. The relevant primers and probe are given in Table 3. The probe was synthesized by IDT (Integrated DNA Technologies).

Ligation of padlock probe

The ligation reaction was as previously described by Wang et al. (2005). One pmol of purified amplicons from16S-23S intergenic was mixed with 2 U of pfu DNA ligase (Stratagene, Integrated Sciences) and 0.1 µm padlock probe in 20 mM Tris–HCl (pH 7.5), 20 mM KCl, 10mM $MgCl_2$, 0.1% Igepal, 0.01mM rATP, 1mM DTT with total reaction volume of 10 µL. Multiple cycle ligations were conducted with one cycle of 5 min at 94°C followed 5 cycles of by 94°C 30 s and 4 min ligation at 60°C.

Table 2 - Nucleotide sequences analysed for padlock probe design.

Id. Name	Nomenclature	Gene/Region Analysed
Streptococcus salivarius	ATCC 9759	rDNA /16S-23S
Streptococcus sanguinis	SSI1655/99	rDNA /16S-23S
Streptococcus ratti	ATCC19645, CCUG 27642	rDNA /16S-23S
Streptococcus criceti	ATCC19642, 24472-01, 24327-01, 26688-01, 23537-01	rDNA /16S
Streptococcus pyogenes	ATCC13540	rDNA /16S
Streptococcus sanguinis	GumJ19, GRTE15B, TeJ10, ChDC OS38, TTE17, ChDC B203, TeTG, RY053, SA049	rDNA /16S
Streptococcus sobrinus	ATCC33478, TT020 WWC_C5ALM116, WWC_C5MLM034, JB026, JC064	rDNA /16S
Streptococcus salivarius	FP051, RW018, FP002, FP045, FP008, FQ015, FQ067, NK037, EQ075	rDNA /16S
Streptococcus mutans	ATCC25175, UC013, UC023, UC003, TT062, TT061	rDNA /16S
Enterococcus faecalis	UPAA100, UPAA102, FUA3333, CD1, EF608, Taj-KH 119, Probio-53, TX0102	rDNA /16S

Rolling circle amplification (RCA) reaction and visualization of amplicons

RCA reactions were performed in a 50 µL volume containing 8 U of Bst DNA polymerase (New England Biolabs), 400 µM deoxynucleoside triphosphate mix, 10 pmoL of each RCA primer. Circularized probe signals were amplified by incubation at 65°C for 60 min. The accumulation of dsDNA products was visualised on a 1.0% agarose gel to verify the specificity of probe-template binding. Positive reactions showed ladder-like pattern, whereas negative reactions demonstrated a clean background.

RESULTS

The padlock probe species-specific based on intergenic region sequences 16S-23S gene of cariogenic bacteria *S. mutans* was designed (Table 3).

Table 3 - Padlock probe sequences and primers used.

Probe/primers	Sequences (5′-3′)
Probe-SM	5PO4' GTAAAAGCCCTATAGCGCAGATCATGCTTCTTCGGTGCCCATGAGGTGCGGATAGCT*CGCGCAGACACGATA*GTCTAGTTCATTGACAATTGAATAGCTA
Primer-1	ATGGGCACCGAAGAAGCA
Primer-2	CGCGCAGACACGATA

- The bold sequences are the binding arms of the padlock probes. Padlock probes are joined by the non-specific linker region.
- RCA primer-1 binds to the padlock probe, generating a long ssDNA. Its sequence is the same as the underlined segments, in reverse.
- RCA primer-2 binds to the nascent ssDNA as their binding sites become available. Each bound reverse primer extends and displaces the downstream primers and their extended products. Its sequence is the same as that of the segments shown in italics.

The padlock probe was evaluated within different strains of *Streptococcus* isolated from the saliva (n=20) from dental biofilm (n=15) obtained from the patients with a high historic of caries; one strain obtained from decayed dentin and one from oropharynx (Table 1). The results were positive for all the DNA samples of *S. mutans* and negative for *S. sobrinus* (ATCC33478 strain) and *S. pyogenes* (ATCC13540 strain) evidencing the specificity of designed padlock probe (Fig. 1).

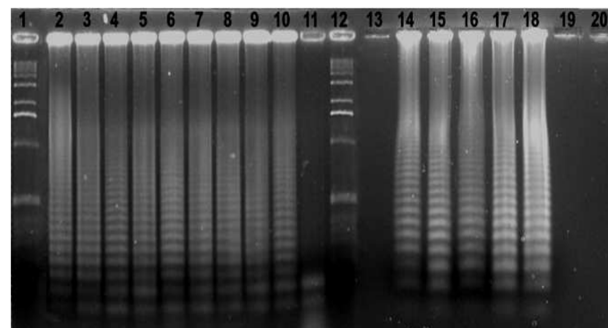

Figure 1 - Analysis of RCA products by agarose gel electrophoresis 1.0%: 01- Molecular Marker 100 bp; 02 to 11- DNA sample of *S. mutans* LB.SM2 to LB.SM11; 12 Molecular Marker 100 bp; 1- Blank control; 14 and 15 – DNA sample of *S. mutans* LB.SM1 and LB.SM12; 16- Total biofilm DNA sample; 17– Total DNA saliva sample; 18– DNA sample *S. mutans* (ATCC 25175 strain); 19- DNA sample *S. sobrinus* (ATCC33478 strain); 20- DNA sample *S. pyogenes* (ATCC13540 strain).

DISCUSSION

The most cariogenic bacteria in dental biofim and oral cavity are *S. mutans* (Hoshino et al. 2004).

Common methods of detection and characterization of bacteria from the oral cavity, especially culture methods, are limited in their sensitivity and specificity. Therefore, molecular biology methods have been developed to overcome culture limitation. Species specific sequences of the 16S rRNA gene has been used for the identification of streptococcal species (Igarashi et al. 1996; Igarashi et al. 1998; Hassan et al. 2001; Hassan et al. 2003; Al-Ahmad et al. 2006). Several sets of primers for PCR have been designed to amplify specific regions of the 16S rRNA genes of *S. mutans* (Yano et al. 2002; Yoshida et al. 2003; Chen et al. 2004) or closely species (Tung et al. 2007). In order to explore the use of molecular biology, 16S and 23S rRNA genes have been used as the targets for identification of microorganisms at the species, genus or family level. These genes contain both conserved regions and areas of variability sufficient for specific identification of bacteria (Hassan et al. 2003).

In this study, in order to discriminate *S. mutans* from the other oral *Streptococcus*, both regions were analysed the 16S and 16S–23S rDNA intergenic spacer regions (ISR). Among the regions examined (Table 2), the 16S–23S rDNA-ISR showed nucleotide changes compatible with the proposed method that was chosen as a target region. The padlock probe based on the rolling circle amplification assay was developed for highly specific and sensitive detection of *S. mutans*. The results were positive for all the 35 DNA samples of *S. mutans* strains evaluated (Table 1), including *S. mutans* ATCC 25175 (Fig. 1), which demonstrated the specificity of designed padlock probe. Furthermore, different samples were tested, including DNA of *S. mutans* isolates from the saliva, from dental biofilm obtained from patient with high historic of caries. The DNA samples of the *S. sobrinus* ATCC33478 strain and *S. pyogenes* ATCC13540 strain were used as negative control (Fig. 1). Results showed that this RCA padlock probe was useful for discrimination of *S. mutans* in biological samples.

The RCA technique has been widely used, with various applications based on this method, such as DNA detection and genotyping (Zhao et al. 2008). This is due the fact that unlike normal PCR, this technique is characterized by an isothermal DNA amplification method and does not need any special equipment for the amplification reaction, being cost-effective and with a low risk of cross-contamination from amplicons associated to highly sensitivity. Therefore, the use of padlock probes based on RCA techniques pose a great potential in clinical diagnosis (Tong et al. 2007; Najafzadeh et al. 2011; 2013; Lackner et al. 2012; Zou et al. 2012; Feng et al. 2013; Hamzehei et al. 2013). This also has been demonstrated the use to identify target nucleic acid sequences, with high specificity down to the single nucleotide polymorphism level- SNPs (Tong et al. 2007).

The use of padlock probes offers advantages over other techniques for detecting the species. A padlock probe comprises two sequences complementary to the 5′ and 3′ termini of the target sequence joined by a genetic linker region. When they hybridize, head to tail, to the target, the 5′ and 3′ ends of the probe are juxtaposed, forming a closed, circular molecule following incubation with a DNA ligase (Nilsson et al. 2006). The ability of padlock probes to accurately identify the sequence target has been demonstrated and the intensity of the signal generated by the circularized probe can be increased exponentially by rolling the circle amplification (RCA) (Nilsson et al. 2006; Tong et al. 2007).

Among hundreds of bacterial species in the human oral cavity, *S. mutans* plays a key role in the development of virulent biofilms of dental caries (Beighton 2005). However, the classical microbiological cultures method limits the studies on the identification of specific populations of *S. mutans* due to over 100 recognized species in the genus *Streptococcus* (Nobbs et al. 2009). Therefore, the development of species-specific padlock probes used in combination with rolling circle amplification for *S. mutans* can be used for a rapid and effective identification of this species, helping towards epidemiologic and diagnostic studies. This Padlock probes developed based on the genetic variability encountered in the flanking regions of the 16S rRNA and 23S rRNA gene seemed to be an appropriate tool to distinguish *S. mutans* strains. Furthermore, this technique could be applied to differentiate the strains and serotypes with different virulence potential favoring the studies about caries disease epidemiology.

In summary, this work revealed that the padlock probe based on the rolling circle amplification assay was highly specific, accurate and useful method for *S. mutans* detection using either DNA of isolates or DNA samples recovered from the biofilm and saliva. A potential future application

would be the detection of specific DNA in clinical samples without preceding the isolation of the bacteria concerned. This was the first report on padlocks probes for *S. mutans*, which appeared potential and promising screening tool for the specific identification of *S. mutans*.

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