

## Methodological Variations in the Isolation of Genomic DNA from *Streptococcus* Bacteria

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### ABSTRACT

*In this work, genomic DNA of Streptococcus pyogenes, S. mutans and S. sobrinus was isolated using two methods: either using the detergent cetyltrimethylammonium bromide (CTAB) at 65°C; or by applying ultrasound to a mixture of silica and celite in CTAB. The composite method that used ultrasound was the more efficient, allowing the straightforward extraction of genomic DNA from Gram-positive bacteria with good quality and reproducibility.*

**Key words:** Key words: *Streptococcus*, DNA isolation, CTAB, ultrasound

### INTRODUCTION

Pyogenic *Streptococcus* belonging to Lancefield's A, C, and G groups are pathogenic agents of a variety of human and animal diseases (Bert et al., 1997). *Streptococcus pyogenes* (*Streptococcus*  $\beta$ -hemolytic of the A group - GAS) is responsible for causing diseases such as erysipelas, pharyngotonsillitis, and scarlatina (Gonzalez et al., 2003), and some serotypes are associated with nonsuppurative complications such as post streptococcal glomerulonephritis and rheumatic fever (Edwards et al., 2003). *Streptococcus* of the Mutans group is usually considered as the main etiological agent of dental caries. This group of microorganisms comprises seven distinct species, including *S. mutans* and *S. sobrinus*, which are most commonly associated with human diseases (Hamada and Slade, 1980; Loesche, 1986).

Advances in molecular biology have provided techniques based on PCR (polymerase chain reaction) and DNA sequencing that allow for improvement in the diagnosis and monitoring of diseases caused by the microorganisms, as well as in epidemiological and transmission studies. Bacterial DNA can be amplified by PCR and then sequenced to provide bacterial identification. An efficient DNA extraction method that results in pure, high quality extracts is important for the success of PCR and subsequent DNA sequencing (Smith et al., 2003), as well as other methods used in molecular biology. The demand for molecular diagnostic tests in medical and oral microbiology underscores the need for efficient methods of DNA extraction.

In order to be applicable in laboratory practices, the DNA extraction process should preferably be inexpensive, safe, simple, reproducible, and

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adequate to the studied samples. The cell wall of Gram-positive bacteria can be broken by the use of enzymes that degrade peptidoglycans such as the lysozyme and mutanolysin present in the commercial kits (Yano et al., 2002; Old et al., 2006), which cause the method to be expensive. The use of cheaper alternative methods is not always appropriated given the difficulty in breaking the cell wall when some reagents are used, such as phenol-ether (Rantakokko-Jalava and Jalava, 2002).

In this sense, bacterial DNA can be extracted from sera by boiling without using any toxic reagent, overcoming the use of expensive commercial kits for DNA purification (Mayoral et al., 2005). Some researchers have already used cetyltrimethylammonium bromide (CTAB) with good results and considerable cost reduction (Doyle and Doyle, 1987; Petersen and Scheie, 2000; Igarashi et al., 2001), at times being combined with sodium dodecyl sulfate (Dellaporta et al., 1983). In 1956, studies by Slade and Vetter have shown that physical methods such as the use of ultrasound or agitation using glass beads facilitates breaching the cell wall.

The goal of the present study was to assess the efficacy and applicability in daily laboratory routine of two methods for the extraction of Gram-positive bacterial genomic DNA, such as *S. pyogenes*, *S. mutans*, and *S. sobrinus*.

## MATERIAL AND METHODS

### Samples

Forty isolates of *S. pyogenes* collected from the oropharynx of employees of a food processing company in Curitiba, State of Paraná, where an outbreak of streptococcal pharyngitis occurred in 2004, and 64 isolates of *Streptococcus* of the Mutans group (*S. mutans* and *S. sobrinus*) obtained from saliva samples of 40 individuals belonging to 9 underprivileged families from the suburbs of Curitiba, PR. All *Streptococcus* isolates were cultivated in brain heart infusion (BHI) broth and incubated at 37°C for 24h. The obtained cultures had approximately  $10^8$  cells, according to MacFarland's scale.

### Total DNA extraction

1. Protocol using CTAB according to Petersen and Scheie (2000), modified. The culture was centrifuged at 49000 g for 2 min, and the

supernatant was discarded. The bacterial cells were washed twice using a TRIS-EDTA (TE) buffer. A volume of 600 µL of CTAB was added to the precipitate, and the entire solution was kept in a heat bath at 65°C for 30 min. After the addition of 800 µL of a chloroform/isoamyl alcohol solution (CIA, 24:1, v/v), the mix was centrifuged at 49000 g for 7 min. A total of 450 µL of the supernatant was removed and transferred to another vial, to which an equal amount (450 µL) of CIA was added, and the solution was homogenized. After centrifuging at 49000 g for 7 min, 300 µL of the supernatant were transferred to another vial, to which 2.5 parts of cold 96% ethanol were added. The vial was left overnight at -20°C. The final step was repeated with cold 70% ethanol, the mix was centrifuged at 49000 g for 7 min, and the vial was left open in a wind tunnel chamber to dry the precipitate. The DNA was then suspended in 50 µL of ultra pure water and stored at -4°C.

2. Protocol using ultrasound combined with silica and celite in CTAB, according to De Hoog et al. (2004), modified: The samples were previously inoculated in BHI broth and incubated for 24h at 36°C. After that the cultures were centrifuged at 49000 g for 2 min and the precipitate was transferred to a vial containing a mixture of powdered silica (silica gel, Merk) and celite (2:1) for maceration in 600µL of CTAB buffer. Three 30s pulses of ultrasound (power 70) were applied to it, with 30s intervals between each pulse, with the vial in ice, using an ultrasonic cell disruptor (Unique ®). After this treatment, 400 µL of CTAB were added to the samples, which were incubated on a heat bath at 65°C for 10 min. After reaching room temperature, 1000 µL of a CIA solution were added to the mix, which was centrifuged at 49000 g for 7 min. The supernatant was transferred to another vial, to which an additional 1000 µL of CIA was added, and was centrifuged again. Approximately 2000 µL of cold 96% ethanol were added to the supernatant, and the vial was incubated at -20°C overnight for the precipitation of the nucleic acids. After this period, the vial was centrifuged at 49000 g for 7 min. The supernatant was discarded and the precipitate was washed with cold 70% ethanol and centrifuged at 49000 g for 7 min. The ethanol was removed and the tubes remained opened in a wind tunnel chamber until the precipitate was completely dry. The precipitate was resuspended on 50µL of ultra

pure water and was left at room temperature for 24h until being stored at -4°C.

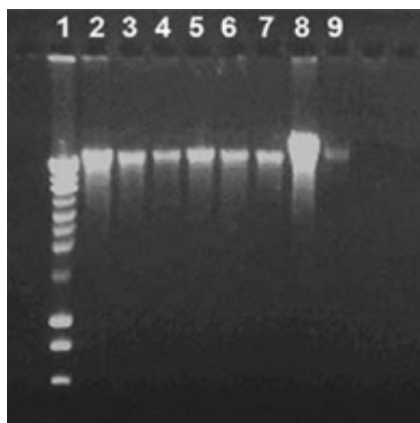
#### Analysis of the DNA extracts

The DNA was quantified using spectrophotometry (260 nm), and its integrity was assessed through 0.8% agarose gel electrophoresis, followed by staining with ethidium bromide and visualization under UV light.

## RESULTS AND DISCUSSION

The results indicated that the efficacy of the DNA extraction of the isolates of *S. pyogenes* as well as the species from the Mutans group (*S. mutans* and *S. sobrinus*) were very different. The method using CTAB at 65°C resulted in the extraction of genomic DNA of only one sample of *S. pyogenes* and 24 samples of isolates from the Mutans group (2.5 and 37.9%, respectively). However, the method using the combination of silica and celite under ultrasound allowed for the extraction of

100% of the isolates of *S. pyogenes* and 91% of the *Streptococcus* from the Mutans group (Fig.1). The CTAB method was described for fungi and plant DNA extraction, and proved successful also for streptococci (Petersen and Scheie, 2000). Despite the success of some researchers in the isolation of bacterial genomic DNA using the CTAB method (Doyle and Doyle, 1987; Petersen and Scheie, 2000; Igarashi et al., 2001), the action of this detergent at 65°C alone was not efficient for breaking the cell wall of the three *Streptococcus* species tested in the present study. However, the protocol using ultrasound combined with silica and celite in CTAB was much more effective in the extraction of genomic DNA of these Gram-positive species. The use of ultrasound increased considerably the efficacy of the breaking of the cell wall, given that the exposition to sonic oscillation of a suspension containing cells of *Streptococcus* caused their cell walls to disintegrate, releasing the cell contents (Slade and Veter, 1956).



**Figure 1** - Extraction of DNA from *Streptococcus*: 1- ladder 100bp, 2, 3, 4 - *S. pyogenes* (300ng, 150ng, 100ng, respectively) 5, 6, 7 - *S. mutans* (200ng, 150ng, 150ng respectively); 8, 9 - *S. sobrinus* (500ng, 50ng, respectively).

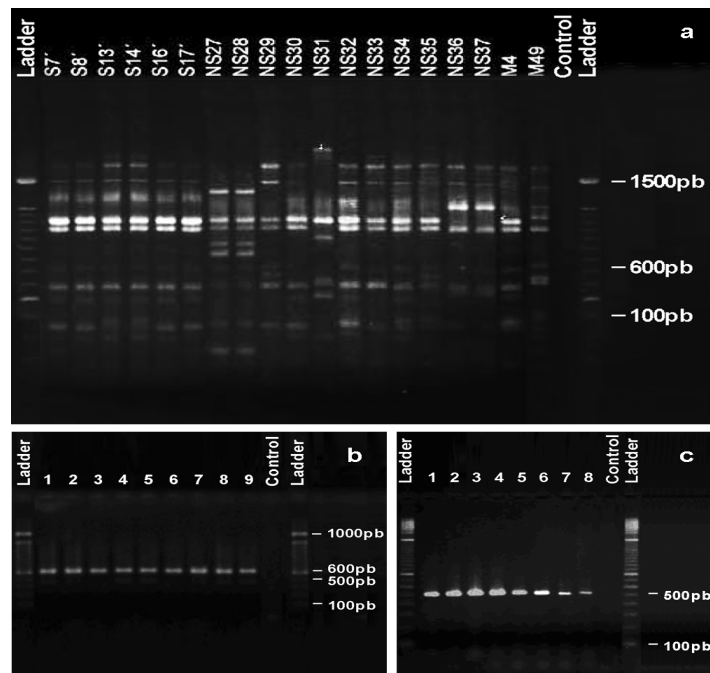
The DNA obtained by using the physical method of maceration with silica-gel together with ultrasound represented a methodological alternative, in which the quantity and quality of the extracted material was sufficient for sequencing or PCR (Fig. 2).

This work demonstrated that molecular detection of bacteria with resistant cell walls in clinical samples could be enhanced by the use of physical cell wall desintegration methods as related by

others authors (Rantakokko-Jalava and Jalava, 2002). Results also indicated that no single method was optimal for the detection of all bacteria that might be present in biological fluids, and the use of more than one method should be recommended. Simplified DNA extraction methods for emm typing of group A streptococci (GAS) have been proposed using heating and thawing in association with the use of mutanolysin (Sigma, St. Louis, Mo.) and hyaluronidase (Sigma) (Beall et al.,

1996; Jose and Brahmadathan, 2006). Both methods were shown to be efficient, given that the extracted DNA was used in different molecular

methods. However, these methods use enzymes, which increases the costs in comparison with the methods used in the present study.



**Figure 2** - Representative gel pictures of the bacterial genomic DNA amplified obtained by the method of ultrasound in association with the mixture of silica and celite in CTAB: a) Randomly amplified polymorphic DNA (RAPD) profile of Group A *Streptococcus* (GAS); b) IGS region amplification profile obtained from genomic DNA of *S. mutans* strains (1-9) c) Sequencing reaction of glycosyltransferase gene (GTS- $\beta$ ) of *S. mutans* strains (1-8).

The advantage of using the physical method as pretreatment without heating or freezing/thawing not even the later use of enzymes, is its simplicity and low cost. In addition, it is useful to standardize these methods for easy and rapid extraction of DNA directly from the isolated colonies of GAS strains and *Streptococcus* of the Mutans group cultivated in liquid media, facilitating molecular studies (Moreira et al., 2007).

The present results supported the assumption that the method using ultrasound with a mixture of silica and celite in CTAB had good reproducibility, was straightforward to execute, and allowed the extraction of high-quality DNA from Gram-positive cells, and, thus, could be used in molecular biology laboratories.

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## RESUMO

O gênero *Streptococcus* encontra-se amplamente distribuído na natureza e algumas espécies constituem a microbiota humana da cavidade bucal, como *Streptococcus pyogenes*, que pode estar associado a importantes doenças humanas, *Streptococcus mutans* e *Streptococcus sobrinus*, relacionados à cárie dental. O DNA genômico destas três espécies foi isolado utilizando-se dois

métodos, o primeiro utilizando o detergente brometo de cetiltrimetilamônio (CTAB) à 65°C e outro associando ultra-som a uma mistura de sílica e celite em CTAB. O método que possibilitou a extração do DNA genômico das bactérias Gram positivas, com qualidade, boa reprodutibilidade fácil execução foi aquele que utilizou ultra-som associado à sílica e celite em CTAB.

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