https://doi.org/10.54589/aol.35/1/51

Saliva sampling methods. Cariogenic streptococci count using two different methods of saliva collection in children

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ABSTRACT

The aim of this study is to compare the efficacy of two methods for collecting saliva samples from infants under 2 years of age for cariogenic streptococci (CS) count. Two collection methods were applied in 11 infants. In Method (A), saliva samples were collected by swabbing the inner cheek mucosa and floor of the mouth in figure of eight motions with a sterile cotton swab until it was soaked. In method (B), saliva samples were collected by aspiration of 1 ml of saliva with a sterile plastic syringe on the floor of the mouth, after stimulation with glove. The samples were cultured in modified Gold's broth (MSMG), and on trypticase, yeast extract, sucrose, cystine and bacitracin culture medium (TYSCB). In method (A), the swab with the sample was unloaded in situ on TYSCB and placed in PBS medium for transport. Then, 100 µl of the eluate was seeded in MSMG. In method (B) 100 µl were seeded in TYSCB and 100 µl in MSMG. Both culture media were incubated under capnophilic conditions for 48 hours at 37 °C. Colony forming units (CFU/ml) were counted by calibrated operators (kappa = 0.75). The presence of cariogenic streptococci (CS) (Streptococcus mutans-Streptococcus sobrinus) was determined by qPCR in the samples collected by both methods. The CFU/ml counts in MSMG differed significantly between methods (p = 0.021). In TYSCB, the recovery of CFU/ml was higher in method (A), without significant difference (p = 0.705). The molecular technique detected presence of CS, with no difference between collection methods.

Collecting saliva samples by swabbing proved more effective in terms of recovery of microorganisms, and did not affect the detection of presence of CS by molecular techniques..

Keywords: *Saliva - Streptococci mutans - Streptococci sobrinus - Infants.*

Recuento de estreptococos cariogénicos a partir de dos métodos de obtención de saliva en niños

RESUMEN

El objetivo de este estudio es comparar la eficacia de dos métodos de obtención de muestras salivales, en infantes menores de 2 años para el recuento de estreptococos cariogénicos (EC). Se aplicaron dos métodos de recolección en 11 infantes, el método (A), consistió en la recolección de muestras de saliva con hisopos de algodón estériles, realizando movimientos en ocho sobre la mucosa del carrillo y piso de boca, hasta embeber el hisopo. En el método (B) la recolección de las muestras se realizó por aspiración con jeringa plástica estéril en piso de boca hasta obtener 1 ml, luego de estimulación con guante. Las muestras fueron cultivadas en caldo de Gold modificado (MSMG) y medio de cultivo TYSCB (tripticasa, extracto de levadura, sacarosa, cistina y bacitracina). En (A), el hisopo con la muestra fue descargado in situ en TYSCB y colocado en medio de transporte PBS. 100 µl del eluato se sembró en MSMG. En (B) 100 µl fueron sembrados en TYSCB y 100 µl en MSMG. Ambos medios de cultivo fueron incubados en condiciones de capnofilia por 48 hs. a 37°C. El recuento de unidades formadoras de colonias (UFC/ml) se realizó por operadores calibrados (kappa= 0.75). La presencia de EC (Streptococcus mutans - Streptococcus sobrinus) fue determinada por qPCR en las muestras obtenidas por ambos métodos. Los resultados mostraron que los recuentos de UFC/ml en MSMG presentaron diferencias significativas entre ambos métodos (p=0.021) En TYSCB la recuperación de UFC/ml fue mayor en el método (A), sin observarse diferencias significativas (p=0.705). Se detectó la presencia de EC por técnica molecular, sin mostrar diferencias entre los métodos empleados.

La recolección de muestra de saliva con hisopo presentó mayor eficacia en términos de recuperación de microorganismos, sin alterar la detección de presencia de EC por técnicas moleculares.

Palabras clave: *Saliva - Streptococcus mutans - Streptococcus sobrinus - Infantes.*

To cite:
Cornejo CF, Salgado PA, Molgatini SL, Gliosca LA, Squassi AF. Saliva sampling methods. Cariogenic streptococci count using two different methods of saliva collection in children. Acta Odontol Latinoam. 2022 Abr 31;35(1):51-57. https://doi.org/10.54589/aol.35/1/51

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Received: August 2021;
Accepted: February 2022

INTRODUCTION

The oral cavity is an easily accessible site from which to collect biological material for studying and diagnosing systemic and oral diseases, and analyzing of microbial communities[1]. Microbial counts in specimens of saliva and dental plaque have provided information for estimating caries risk in adults and children, showing a positive correlation between caries experience and cariogenic streptococci (CS) counts[2]. The methods proposed for counting bacteria include culture in selective media, biochemical tests, immunological and genetic tests with DNA probes, enzyme-linked immunosorbent assay, and polymerase chain reaction (PCR)[3]. Culture in selective media is a useful tool for morphotyping, counting colony-forming units (CFU/ml) and obtaining bacterial strains for molecular processes to enable genomic studies. Media such as Mitis Salivarius Agar (MSB) and Tryptic Soy Agar (TSY20B) are often used in studies to demonstrate the correlation between cariogenic streptococci counts and caries lesions[4]. Gliosca et al.[5] proposed another technique for counting bacteria by means of an adhesion test (AA-MSMG) using the selective culture medium modified Gold's broth (MSMG-20% sucrose) to evaluate cariogenic risk. This method has satisfactory predictive value and is a valid instrument to categorize patient risk. In addition, molecular techniques such as real-time polymerase chain reaction (qPCR), due to their high sensitivity, specificity and speed, are also effective for detecting and quantifying bacterial species[6].

Regardless of the microbiological analysis methods, saliva sampling needs to be performed using simple, affordable, noninvasive techniques, which are standardized, valid and reproducible.

Some salivary components vary according to the saliva collection method. Therefore, before beginning with a study, a technique must be selected to optimize sample collection according to the study objective and/or the biomarker to be analyzed[7], taking into account the type of saliva to be collected (stimulated or unstimulated) and the context in which the study is to be performed. Results will thus be comparable to those of other studies.

Saliva samples can be obtained using absorbent or non-absorbent methods. Absorbent methods include collecting samples using different materials such as paper strips, cotton rolls and polystyrene and polyethylene swabs. Non-absorbent methods include collecting biological material by spitting or passive drooling into sterile collection containers, and sampling by aspiration with devices such as plastic syringes, among others[8,9].

There are some studies evaluating the efficiency of saliva collection methods for studying different biomarkers[10,11]; however, they have not been validated for detecting and identifying microorganisms in children.

The aim of this study is to compare the efficacy of two methods for collecting saliva samples from infants under two years old for cariogenic streptococci (CS) count. Microorganisms were counted using two differential selective culture mediums and by molecular detection.

MATERIALS AND METHODS

This study was conducted on 11 infants aged 6 to 28 months who attended an early childhood center in Buenos Aires City. This was part of the project "Horizontal transmission and early colonization of mutans group Streptococci in infants who attend mother-and child educational centers", approved by the Ethics Committee at the Buenos Aires University School of Dentistry (CUDAP: EXP-UBA: 0072332/201 7 Nº O12/2018 C.ÉTICA FOUBA).

Saliva specimens were collected from each participant using two different methods: an absorbent method (A), and a non-absorbent method (B). The methods were applied in alternative order on different infants to avoid the possibility of the second method collecting a smaller specimen due to the child being more tired or less cooperative.

In Method (A), saliva samples were collected by swabbing the inner cheek mucosa and floor of the mouth in figure of eight motions with a sterile cotton swab until it was soaked. Then the swab was unloaded in situ by plating on Petri dishes containing TYSCB (Tryptone Yeast Extract Cystine Sucrose and Bacitracin) culture medium, and then in Eppendorf-type tubes containing phosphate-buffered solution (PBS), for transfer. In method (B), saliva samples were collected by aspiration of 1 ml of saliva with a sterile plastic syringe on the floor of the mouth, after stimulation with glove. The content of the plastic syringe was unloaded in Eppendorf-type tubes. The samples were taken to the Microbiological Diagnosis Laboratory at Buenos Aires University's School of Dentistry within 2 hours of sampling.

The saliva samples were vortexed and seeded in two differential selective culture mediums for colony-forming unit count (CFU/ml).

The samples taken using method A were plated in TYSCB (in situ), and 100ul of the elute in PBS was seeded in modified Gold's broth (MSMG)[5]. Equivalent aliquots of the samples taken using method B were seeded in TYSCB and MSMG.

Cultures were incubated under anaerobiosis (GasPack - Mitsubishi®) for 48 hours at 36 ºC ± 1 ºC. After incubation, calibrated personnel (Kappa >0.75) observed the cultures under stereoscopic microscope ("Arcano" ST30-L binocular stereo microscope) at 50X magnification. Counts were performed considering the morphological characteristics of the colonies described for cariogenic streptococci under the study conditions. On TYSCB, counts were performed up to a maximum 300 characteristic colonies per plate, and values higher than this were not recorded. Adhered colonies applying the AA-MSMG were counted in 3 areas of 1cm^2, using a grid covering the entire contact the area of the culture bottle.

Molecular processing to determine cariogenic streptococci (Streptococcus mutans – Streptococcus sobrinus) was performed using species-specific primers for real-time polymerase chain reaction (qPCR) method. The genomic material from the samples from methods A and B was obtained by following the instructions provided by the manufacturer of the commercial kit (Presto™ Mini gDNA Bacteria Kit, Geneaid). DNA integrity was quantified and evaluated by spectrometry (CYTATION 3 Cell Imaging reader, Biotek). The DNA samples employed had values between 1.7 and 2.0 (ratio 260/280 nm) and their concentrations were normalized at 20 ng/microliter.

Detection and quantification of Streptococcus mutans (S. mutans) and Streptococcus sobrinus (S. sobrinus) were performed by qPCR in a CFX96TM Real Time System thermocycler (Bio-Rad Laboratories, Inc.). Species-specific primers were employed, using as a target the gene encoding the following glycotransferase enzymes: gtfB, for S. mutans and gtfT S. sobrinus[12]. The reactions were performed in triplicate, using Sso Advanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Inc.) in a final volume of 10 microliters.

Statistical processing consisted of calculating the mean rank for each collection method and each culture medium, and comparing the results by two-way analysis of variance by ranks (Friedman's test). For the molecular technique, the percentages of positive results for S. mutans and S. sobrinus for each collection method were calculated, and differences were analyzed using McNemar's test.

RESULTS

Seven females and four males took part in this test. Average age was 14.9 months (SD±4.67).

Mean rank for the CFU/ml count for AA-MSMG was 1.83 for method A, and 1.17 for method B (Fig. 1), with statistically significant differences (p=0.021).

Mean rank for the count on cultures on selective TYSCB medium was 1.54 for method A, and 1.46 for method B, without statistically significant difference (p=0.705) (Table 1), though recovery was greater in method A (Fig. 2).

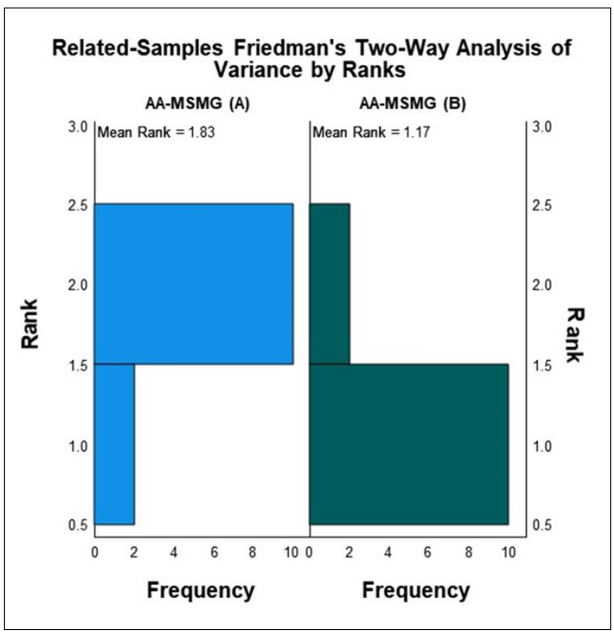

Fig.1: Mean rank of CFU/ml according to a sampling method. AA-MSMG: Culture on Gold Broth modified with 20% of sucrose (A): absorbent method, (B): non-absorbent method.

Table 1. Cariogenic streptococcus count in TYSCB for collection methods A and B

	Median	Percentile 25	Percentile 75	p
TYSCB (A)	158	0	300	0.705
TYSCB (B)	6	2	154	

TYSCB: Culture in Tryptone Yeast Extract Cystine Sucrose and Bacitracin medium. (A): Absorbent method, (B): Non-absorbent method.

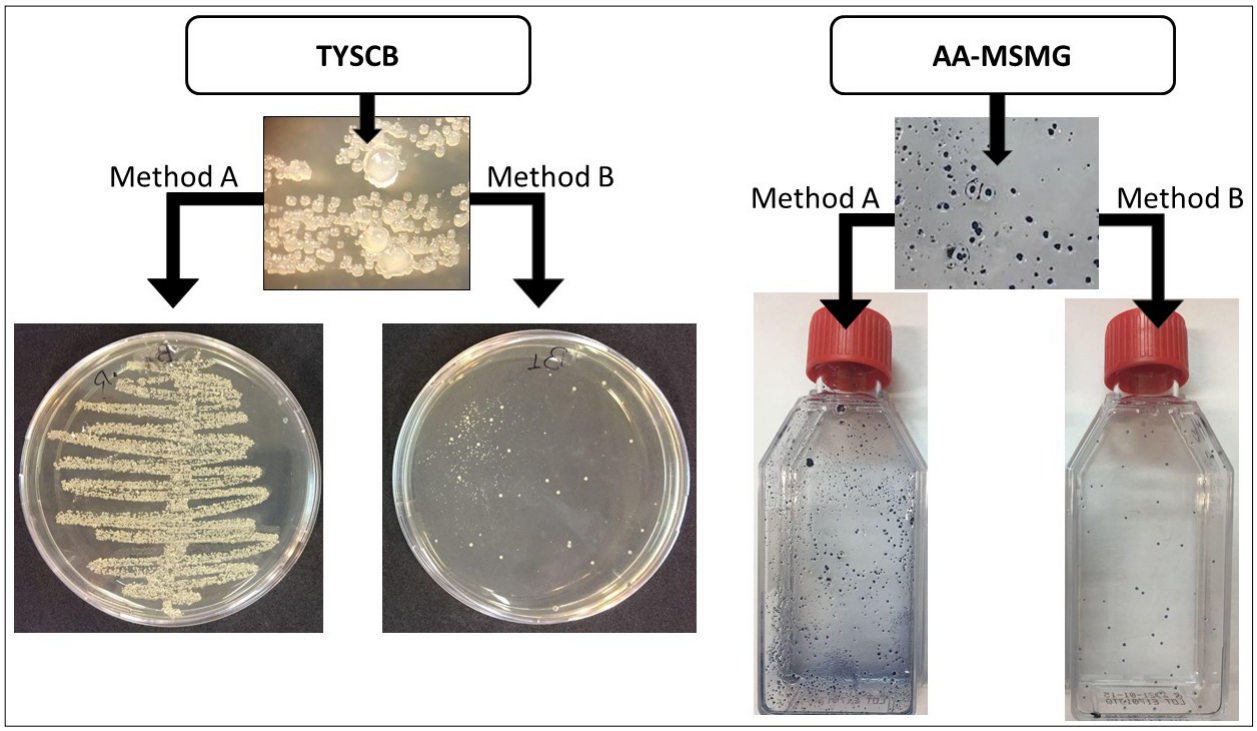

Fig.2: Culture on Tryptone Yeast Extract Cystine Sucrose and Bacitracin (TYSCB) and Gold Broth modified with 20% sucrose (AA-MSMG). Method A = absorbent. Method B = non-absorbent.

With qPCR, positive results for S. sobrinus and S. mutans were 36.4% and 45.5%, respectively, for method A; and 75% and 41.7%, respectively, for method B. For qPCR, no significant difference was found between sampling methods (S. sobrinus: p=0.21; S. mutans: p=0.941) (Table 2).

Table 2 - Detection of cariogenic streptococcus by qPCR using collection methods A and B

Method	Positive	% Positive	p
S. *sob* qPCR A	4	36.4%	0.219
S. *sob* qPCR B	9	75.0%	
S. *m* qPCR A	5	45.5%	0.941
S. *m* qPCR B	5	41.7%	
qPCR A	8	72.7%	1.000
qPCR B	10	83.3%	

McNemar Test
S. sob = Streptococcus sobrinus, S. m = Streptococcus mutans

DISCUSSION

Microbiological studies on saliva specimens are needed to establish cariogenic risk level and identify microorganisms that may be associated with the dental caries process. However, taking samples from infants may involve difficulties related to children's cooperation or unfavorable perception of sampling methods by aspiration with syringes.

Our study compared a suction sampling method and an absorbent sampling method, finding that they provided similar results for colony counts and genetic material. The absorbent method enabled greater detection of CFU/ml in the AA-MSMG culture technique.

The absence of significant differences between methods when the count was performed on TYSCB may be explained by the fact that the count parameters established for this method did not assign an exact numerical value when counts were higher than 300 colonies, which was taken as maximum value.

We assume that the differences observed could only be attributable to the intrinsic characteristics of each method, because the samples were taken simultaneously under similar conditions.

This result was in agreement with other reports[14,15] which used swabbing to collect specimens from infants aged 0 to 30 months and 0 to 6 months.

However, Motisuki et al.[13], for stimulated saliva simples in a population of children aged 5 to 13 years, found a significantly lower CS CFU/ml count when samples were collected by swabbing than when collected by methods using dental biofilm or drooling

into sterile collector tubes. This difference may be explained by the fact that our swabbing method applied motion in the infant's oral cavity, which may have caused detachment of microorganisms in the oral cavity during the procedure.

Some studies have compared the ability to detect specific bacteria in unstimulated and stimulated saliva samples collected using different techniques and cultured in different mediums under the hypothesis that the bacterial component in saliva varies according to type of sample collected.

One study on 3-year-olds[16] determined the presence of S. mutans in unstimulated saliva samples collected by soaking a cotton swab under the tongue compared to samples taken by moving a swab around in the oral cavity. It found that presence of S. mutans was higher in oral swab samples than in unstimulated saliva samples. This agrees with the results of our study, which found that the oral swab technique produced higher CS recovery.

Saliva is increasingly being used as a biological material in which to study the oral microbiome. It is therefore necessary to create a protocol for collection methods, considering potential interference factors. A recent study by Omori[17] reports finding similar percentages of relative abundance of streptococci for the drool method and the swab method, recommending the cotton swab method to study the microbiome in subjects who cannot produce saliva or have difficulty in spitting.

There is evidence that stimulated saliva samples could be used as a substitute for unstimulated saliva for oral microbiota studies and that bacterial profile would not vary significantly according to type of saliva specimen used[18]. Another study[19] comparing the composition of oral microbiota between stimulated saliva (with paraffin block) and unstimulated saliva (paper points) found significant differences, with stimulated saliva containing an estimated number of species three times higher than unstimulated saliva.

Different authors have suggested that some absorbent collection devices can introduce bias and errors in the subsequent data analysis, mainly in relation to immunoglobulin assays and studies focusing on certain steroid hormones[20-22]. Our study found that swabbing did not alter the detection of cariogenic streptococci identified by molecular analysis (qPCR), in agreement with other authors[23,24] who established that microbial profiles in saliva are minimally affected by the collection method.

Taking saliva samples in young children is challenging as a result of situations such as the time involved in obtaining specimen volumes large enough for subsequent processing and analysis, compliance with protocols prior to sampling (fluid, food and medication intake, and mouthwash before sampling), sleep cycles, and the child's predisposition to the practice. For example, Granger et al.[25] reported difficulties in collecting saliva from children aged 6 to 15 months related to ethnicity and the socioeconomic level of the families.

Although the aim of the current study was not to establish association between sampling and aspects related to family income level, it is worth noting that the early childhood center where it was conducted is attended by children from low-income families. The center provides meals (breakfast, lunch and afternoon snack), sleep time (30 to 60 minutes to rest) and play activities. These situations justified the performance of this study to identify the most appropriate method for collecting samples in the context.

In developed countries today, there are available commercial devices that are easy to use, cause minimum discomfort to participants, and obtain adequate quantities of specimens for subsequent processing. However, in developing countries, where financial and bureaucratic limitations hamper the purchase of complex devices, the standardization of protocols with low-cost supplies offers alternatives for conducting microbiological studies.

This study shows that the oral swab method for collecting saliva samples is more effective in terms of recovering microorganisms, and does not alter CS detection by molecular methods. It thus provides preliminary evidence contributing to the development of protocols and methods for obtaining saliva specimens from infants.

ACKNOWLEDGMENTS
The authors thank authorities and teachers at "El Alfarero" early childhood center in Buenos Aires City for their participation, and the families of participating infants.

DECLARATION OF CONFLICTING INTERESTS
The authors declare no potential conflicts of interest regarding the research, authorship, and/or publication of this article

FUNDING
This study was supported by grant UBACYT 20620170100011BA, from Buenos Aires University.

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