Validation of an adherence assay to detect group mutans streptococci in saliva samples

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ABSTRACT

The aim of the present study was to validate and establish a cut off point and the predictive value of an adhesion test (AA-MSMG), as a microbiological method for evaluating cariogenic risk. The study is based on a variant (20% sucrose) of a selective medium descripted by Gold et al. (MSMG). This method differentiates mutans group streptococci (MGS) by exacerbating the production of insoluble extracellular polysaccharide which gives adhesion to surfaces such as glass, plastic and dental enamel. Caries assessment according to ICDAS was conducted in 154 patients (aged > 21 years) who were attended at Preventive and Community Dentistry Department, School of Dentistry, University of Buenos Aires, Argentina, between August 2017 to August 2018. The study population was assigned to groups according to the presence/ absence of caries lesions: Group A: ICDAS lesion code = 0 (L=0) on all dental surfaces (n=23); and Group B: L> 1 (n=131). After mouth-rinsing with distilled water, saliva samples were collected with fasting and hygiene protocol, and sent immediately to the Microbiological Diagnosis Laboratory, Microbiology Department, School of Dentistry, University of Buenos Aires. Samples were homogenized and serially diluted to the tenth. 100 µl of the dilutions were cultured in 25 cm^2 sterile plastic flasks containing 9.9 ml of modified selective medium described by Gold (MSMG-selective and differential medium). Cultures were incubated in an anaerobic atmosphere at 36 ± 1ºC for 48 hours. The supernatants were eluted and the samples washed with sterile distilled water. Colony forming unit counts were performed by calibrated researchers (Kappa ≥0.75) using a stereoscopic microscope at 50X.

Mutans group streptococci (MGS) counts ranged from 1x10^4 to 1x10^5 CFU/ml in group A, and were higher than 1x10^6 CFU/ml in Group B.

Statically analysis of results (ROC) showed that the AAMSMG has a satisfactory predictive value (91%) and established a cutoff point in 1.68x10^5 UFC / ml. This would indicate that individuals whose MGS saliva counts are higher than the cutoff value would be 5 times more likely to develop dental caries. Adherence assay could be a useful microbiological predictor of caries risk.

Received: May 2019; Accepted: August 2019

Keywords: *mutans group streptococci; saliva; carious lesions.*

Validación del Test de adherencia para el recuento de estreptococcos del grupo mutans en muestras de saliva

RESUMEN

El objetivo del presente estudio fue validar, establecer el punto de corte y valor predictivo de una técnica microbiológica para evaluar el nivel de estreptococos del grupo mutans en saliva. La técnica consiste en un test de adherencia que emplea un medio selectivo modificado (20% sacarosa) descripto por Gold et al. (TA-MSMG). Este método permite diferenciar a los estreptococos del grupo mutans (SGM) exacerbando la producción del polisacárido extracelular insoluble que le confiere adhesión a superficies como vidrio, plástico y esmalte dental. De acuerdo con los criterios de ICDAS se sembraron 154 salivas de pacientes mayores de edad, que asistieron al Servicio de Odontología Preventiva y Comunitaria de la Facultad de Odontología de la Universidad de Buenos Aires entre los meses de agosto de los años 2017 y 2018.

La población estudiada fue asignada a dos grupos según la presencia / ausencia de lesiones de caries: Grupo A: código de lesión ICDAS = 0 (L = 0) en todas las superficies dentales (n = 23); y Grupo B: L> 1 (n = 131). Después de realizar un enjuague bucal con agua destilada, las muestras de saliva se recogieron según protocolo (ayuno de 4 horas y suspensión de higiene dental de 12 hs). Las muestras se remitieron de inmediato al Laboratorio de Diagnóstico Microbiológico, Departamento de Microbiología de la Facultad de Odontología, Universidad de Buenos Aires. Para su procesamiento, las muestras fueron homogeneizadas y diluidas al décimo. Se cultivaron 100 µl de las diluciones en botellas de plástico estériles de 25 cm^2 que contenían 9,9 ml de medio de Gold modificado (MSMG-20% sacarosa). Los cultivos se incubaron en atmósfera anaeróbica a 36 ± 1ºC durante 48 horas. El

sobrenadante se eluyó y las muestras se lavaron con agua destilada estéril. Los recuentos de unidades formadoras de colonias SGM fueron realizados por investigadores calibrados (Kappa ≥0.75) utilizando un microscopio estereoscópico a 50X. Los recuentos de SGM presentaron una variación entre 1x10^4 y 1x10^5 UFC/ml en el grupo A, mientras que en el Grupo B fueron superiores a 1x10^6 UFC/ml.
El análisis estadístico de los resultados determinó una curva ROC que establece para el TA-MSMG un valor predictivo del 91% y un punto de corte en 1.68x10^5 UFC SGM / ml. Esto indicaría que los individuos cuyos recuentos en saliva de SGM sean superiores al valor de corte, tendrían 5 veces más posibilidades de desarrollar caries (5:1). Este método podría ser un instrumento útil al momento de evaluar (indicador microbiológico) el riesgo cariogénico del paciente.

Palabras clave: *Estreptococos del grupo mutans; saliva; caries.*

INTRODUCTION

Mutans group streptococci - MGS (*S. mutans*– *S. sobrinus*) belong to the indigenous microbiota of the oral cavity. However, given their metabolic characteristics, they are closely associated with caries lesions in humans[1]. Caries, a multifactorial ecological infectious disease, is the result of environmental changes triggered by the interaction of many contributing factors including the environment, bioavailability of substrates (sucrose), tooth surface, and the presence of cariogenic biofilm[2]. The persistence of such particular conditions has been found to result in an increase in the concentration and distribution of mutans group streptococci. The ecological change is evidenced by qualitative and quantitative remodeling of the supragingival biofilm, which favors the production of large amounts of soluble and insoluble extracellular polysaccharides and a marked pH decrease at the saliva-plaque interface. Production of insoluble, mutans polysaccharides on the smooth surfaces of teeth is an undisputed characteristic of sucrose-dependent biofilm, and is a relevant cultural feature of MGS[3].

Supragingival biofilm, which remodels and consolidates through time, is able to withstand environmental changes due to its ability to reorganize and become metabolically autonomous. This is typical of mature biofilm (*Quorum sensing*)[4]. The strategy of preventive dentistry is based on identification and establishment of the elements that will facilitate diagnosis and foreseeing possible short and mid-term changes in the oral environment. To this end, indicators have been developed to determine the likelihood of a patient having the conditions needed for the occurrence of microbiological, ionic, and chemical imbalances that promote demineralization of dental tissues (cariogenic risk – CR). Such indicators include mutans streptococci and *Lactobacillus* spp (LB) counts in saliva[5].

Making clinical decisions is an extremely complex process, and must ultimately rely on the usefulness of a diagnostic test for patient management. It is therefore essential to have detailed knowledge and a thorough understanding of the precision of different available diagnostic tools; in other words, to understand the accuracy of each test in order to classify patients in categories or according to status with regard to the disease. For this purpose, statistical parameters such as sensitivity and specificity of the diagnostic tests are assessed, and the positive and negative predictive values are calculated based on the prevalence of the disease that is to be diagnosed, and are used to determine the usefulness of the tool.

Efforts to obtain a reliable indicator for enumerating salivary mutans streptococci have resulted in the development of different isolation and identification methods using selective and/or differential media[6]. Some commercial kits are used as reference tables (*Cariesscreen*®[7] *Dentocult*SM®[8]), whereas others are diagnostic tests with a set cut- off point.

Clinical microbiology laboratories seek to develop different selective and differential media that allow recovering the largest amount of MGS serotypes occurring in the samples, and significantly diminish the indigenous background microbiota. [9-11] The broth used at our laboratory is a modification of a selective medium designed by Gold for mutans group streptococci [12-15] (20%P/V sucrose SIGMA®) Selectivity and specificity are adjusted to enhance the production of insoluble, or mutant polysaccharides, which are specific to MGS.

The aim of the present study was to validate and establish a cut-off point and the predictive value of an adhesion test (AA-MSMG), as a microbiological method for evaluating cariogenic risk.

MATERIALS AND METHODS

Study Population

The study population comprised 154 male and female patients aged more than 21 years (25.27± 7.25), who attended the Department of Preventive and Community Dentistry, School of Dentistry, University of Buenos Aires between August 2017 and August 2018.

All enrolled patients gave their written informed consent. They were interviewed to obtain information regarding on their diet, oral hygiene habits, frequency of dental visits, and recent medical-dental treatments, and were clinically examined and scored according to the ICDAS-II criteria.[16,17] The patients were then assigned to one of the following groups : Group A (n=23): patients without carious lesions (*ICDAS II, code 0*) (L=0); Group B (n=131): patients with carious lesions (L >= 1) [18].

METHOD

Patients who had received antibiotic therapy or dental treatment within one month prior to sample collection were excluded from the study. The participants were instructed to come fasting and without brushing their teeth on the day of sample collection. Unstimulated saliva was collected by spitting into a falcon tube for 1 minute. The obtained sample was sent immediately to the Microbiological Diagnosis Laboratory, Microbiology Department of the School of Dentistry, University of Buenos Aires and processed in keeping with the biosafety protocols of the Institution. (Resol CD 287/07).

Saliva samples were homogenized by vortexing for 30 seconds and then were diluted to the tenth in phosphate buffer solution (0.01M PBS pH 7). 100µl of the pure and diluted samples were cultured in sterile plastic flasks (25cm^2 contact surface area), containing 9.9 ml of modified selective media described by Gold (MSMG-selective and differential medium with 20% sucrose). The inoculated flasks were placed horizontally and incubated in anaerobic conditions at 36 ±1ºC for 48 hours. Following the incubation period, the flasks were gently inverted several times in order to eliminate the colonies not adhered to the plastic surface. The supernatants were discarded, and the flasks were rinsed twice with sterile distilled water.Colonies were counted by calibrated observers (index Kappa≥ 0,75) in three 1cm^2 fields , randomly distributed on the contact surface of the flasks, using a stereoscopic microscope at 50X magnification. Total counts were corrected taking into account the total contact surface area (25 cm^2), the 100 µl culture aliquot and the dilution factor applied to the sample. Total salivary SGM CFU /ml counts of saliva were thus calculated for each patient.

An MI strain of *Streptococcus mutans (Sm*MI) (Arginine Dihydrolase, Mannitol, Sorbitol, Esculin, Voges Proskauer – ROSCO® tablets and 6.5% sodium chloride – hypertonic broth Britania S.A) from the collection of microbial cultures of the Microbiology Department was used as intra-assay control. The inoculum was adjusted to Nº 0.5 on McFarland's scale, and the culture was processed under the same conditions as the experimental samples. Sensitivity was determined by limiting dilution. In addition, the viability of different strains of indigenous microbiota was tested using a modification of the selective medium developed by Gold *et al* (MSMG). The studied strains were: *Staphylococcus aureus* (*Sa*) ATCC 25923, *Candida albicans* (*Ca*), *Enterococcus faecalis* (*Ef)* ATCC 29212, *Escherichia coli* (*Ec*) ATCC 25922, *S mutans* (*Sm*) ATCC 25175, mutans group streptococci (MGS SmMI) and *Streptococcus sanguinis* (SsMI), wild strains included in our microbial culture collection.

Statistical Analysis

In order to determine the diagnostic value of the AA-MSMG test (sensitivity, specificity, and adequate overall accuracy), the data were statistically analyzed by plotting the Receiver Operating Characteristic curve (ROC curve) using SPSS 25[19].

RESULTS

Although the cultures of the studied strains (*Sa, Ca, Ss*MI, *Ef* and *Ec*) were found to grow in MSMG, none of them adhered to the plastic surface of the flasks as a result of insoluble extracellular polysaccharide production. Total counts, macro and microscopic morphologic features of the *Sm*MI and *Sm* ATCC 25175 strains were consistent with reference values for growth in this medium (Figs 1, 2 and 3). 1 x 10^3 CFU/mL of saliva was the lowest number of GMS that can be detected using this technique. For validation of the AA-MSMG as a diagnostic tool, the data was processed by ROC (Receiver Operating Characteristic)[20] (Figs.4 and 5). Data analysis established 78.63% sensitivity, 56.52% specificity, 91.15% positive predictive

Fig. 1: Polystyrene flask with mutans streptococci adhered colonies.

Fig. 2: Colonies adhered with insoluble extracellular polysaccharide.

Fig. 3: Mutans group streptococci colony with insoluble extracellular polysaccharide.

value (PPV), 31.71% negative predictive value (NPV) (Table 1), 1.68x10^5 UFC/ml cut off point (Table 3), 0.722 areas below the curve (Fig 4), and 4.782 risk to develop caries (Table 2). This method used in a population of 154 people showed that levels of MGS counts higher than the cut-off point (1.68 x 10^5 CFU/ml), increase the microbiological risk of developing caries up to 5 times.

DISCUSSION

The caries process differs according to the genotype of each individual and the environmental stimulus to which the individual is subjected. Nevertheless,

Table 1: Analysis of ROC curve parameters.

		95% C.I.	
		Lower limit	Upper limit
Prevalence of the disease	85.06%	79.44%	90.69%
Correctly diagnosed patients	75.32%	68.52%	82.13%
Sensitivity	78.63%	71.61%	85.65%
Specificity	56.52%	36.26%	76.78%
Positive predictive value	91.15%	85.91%	96.39%
Negative predictive value	31.71%	17.46%	45.95%
Likelihood Ratio +	1.808	1.125	2.906
Likelihood Ratio -	0.378	0.233	0.615

The positive predictive value of the AA-MSMG makes it a reliable method to be used as a population-screening tool

Table 2: ROC curve. Risk estimation parameters.

		95% Confidence interval	
	Value	Low	High
Reason for the advantages for Sm Count (High/Low)	4.782	1.898	12.050
For cohort Presence of caries= Yes	1.335	1.075	1.657
For cohort Presence of caries= No	0.279	0.133	0.587
Number of valid cases	154		

AA-MSMG results in a population of 154 people shown that levels of MGS counts higher than the cutoff point (1.68 x 105 CFU/ml), increase the microbiological risk of developing caries up to 5 times

Table 3: Group mutans streptococci counts obtained using available methods.

Method	Reference Values (CFU/ml saliva)
AA-MMG	**$1.68x10^5$**
Cariesscreen	$1x10^4$-$1x10^5$= low risk $2.5x10^5$-$5x10^5$= moderate risk **>$5x10^5$= high risk**
Dentocult SM	Class 0-1: <$1x10^5$ Class 2: between 0-1 and 3 **Class 3: >$1x10^6$**

Cariesscreen® and DentocultSM® are commercial kits behave as reference tables, whereas the AA-MSMG is a diagnostic test with a lowest set cutoff point.

in view of the multifactorial etiology, world-wide distribution, and high prevalence of dental caries, it is paramount to develop tools that allow measuring the level of infection by microbial agents that are indicators of the disease (MGS – LB)[21], and which can be applied in populations with high social vulnerability in developing countries. The method used in the AA-MSMG involves collecting unstimulated saliva and counting colonies adhered by mutans after serial rinsing, using a stereoscopic microscope.

The method (AA-MSMG -contact surface area) promotes an increase in the microbial specificity in the sample. It allows and enhances insoluble exopolysaccharide (IEPS) production by mutans streptococci and provides the latter with stable and insoluble adherence and cultural features that are specific to colonies producing IEPS. Using serial dilutions facilitates microbial counts by decreasing operator error. Obtaining negative cultures for MSG could mean that ecological balance has been reestablished in healthy proportions.

Commercially available kits show ranges of unspecific cariogenic microbial counts according to tables provided by the manufacturer, and establish a direct correlation with different levels of caries risk. Although the usefulness of the AA-MSMG to assess caries risk *per se* has not been studied to date, the results of the present study suggest that AA-MSMG can be used as a microbiological indicator.

The analysis of the obtained results showed that sensitivity of the AA-MSMG was 78.63% and PPV was 91%. Kigmanet al[22], however, obtained a sensitivity of barely 31% using a similar culture technique in MSB (Mitis Salivarius Bacitracin).[6] The variations in sensitivity could be associated

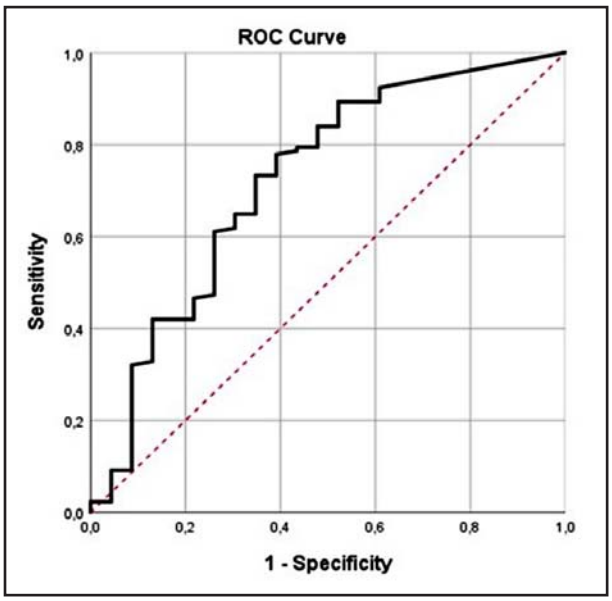

Fig. 4: ROC Curve. Area under the curve:

			Asymptotic 95% Confidence Interval	
Area	Std. Error[a]	Asymptotic Sig.[b]	Lower Bound	Upper Bound
0.722	0.063	0.001	0.598	0.846

the test result variable(s): RTO *Sm* has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.
a. Under the non parametric as sumption
b. Null hypothesis: true area = 0.5

with the methodological characteristics of the test and the use of magnification to perform microbial counts. In comparison with other commercial methods, such as CARIESCREEN®[9], their results are consistent with the cutoff point obtained in the present study using the AA-MSMG.

Studies conducted by Buischi *et al*.[23] in Brazil and by Klock *et al*.[24] in Sweden showed that MGS counts in saliva above 1.10^6 CFU/ml correlated positively with the presence of dental caries. Their results are above the cut-off point obtained in the present study.

Other researchers, such as Sullivan *et al*.[25], have challenged the true predictive value of microbiological indicators (MGS-LB) in comparison with other indicators of caries risk. As is often observed in the literature, microbiological indicators are thought to have poor validity *per se*, and are considered reliable only when they present simultaneously with high microbial counts (MGS) and clinical evidence of caries. In view of their PPV, microbiological

indicator must be considered as an additional parameter that provide information about the level of infection in a patient, and which could possibly modified to contribute to restore the homeostasis of the oral environment. This method has few limitations; it needs trained observers to perform the counts of specific and characteristic adhered colonies, and basic laboratory equipment. Microbiological diagnostic laboratory can employ it because the method no poses severe operative difficulties.

In view of the sensitivity, PPV, ABC and risk values, AA-MSMG would seem to be an adequate cariogenic-screening test. It could be considered as a valid diagnostic test to measure the infection level of a cariogenic microorganisms group such as mutans streptococci. This test will safely estimate the likelihood of an individual developing caries when salivary MGS counts are above the cut-off point. It will reliably measure biological risk factor and contribute to establishing cariogenic risk with greater certainty.

FUNDING
This study was supported by research grants from the University of Buenos Aires (UBACYT 20720160100001BA)

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