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Comparative Analysis of a Monoclonal Antibody-Based *Streptococcus mutans* Detection Method with Selective Culture Assays Using Polymerase Chain Reaction as a Gold Standard

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ABSTRACT

The aim of this study was to compare a recently developed monoclonal antibody (MAb)-based salivary *Streptococcus mutans* detection method with various selective media using polymerase chain reaction (PCR) as the gold standard. Salivary *S. mutans* cells were enumerated with a MAb-based method, along with three commonly used selective media, mitis-salivarius-bacitracin agar (MSB), trypticase yeast-extract cystine sucrose bacitracin agar (TYCSB), and glucose-sucrose-potassium tellurite-bacitracin (GSTB) agar. Statistical analysis showed no significant correlations between each method. With PCR as the standard, a MAb-based detection method was found to provide the highest sensitivity (91%) and specificity (96%) among these four methods. This study demonstrates that the MAb-based detection method may provide more accurate enumeration of salivary *S. mutans* than selective media.

INTRODUCTION

SINCE *STREPTOCOCCUS MUTANS* was first isolated and described by Clark in 1924,[1] it has been considered a major cariogenic bacterium involved in the initiation and progression of dental caries.[2–4] The bacterium was found to induce dental caries in human subjects and experimental animals.[2–5] The correlation between *S. mutans* counts in saliva or dental plaque and the incidence of dental caries has been postulated. Various research groups have explored this association.[6–23] Unfortunately, the results have been inconsistent and not conclusive. While some studies found positive associations,[6,9,11,13,15,20–23] others obtained conflicting results.[7,8,10,12,14,16,18,19] There are many variables in the studies that could be responsible for the different results. One of these could be the selective culture method used in these studies to enumerate *S. mutans*.[24] The most commonly used selective medium for *S. mutans* is mitis-salivarius-bacitracin (MSB) agar.[25] The other selective media include trypticase yeast-extract cystine sucrose bacitracin (TYCSB) agar[26] and glucose-sucrose-potassium tellurite-bacitracin (GSTB) agar.[27] Because there is no selective medium that allows only one bacterial species to grow, these culture-based methods only provide limited accuracy.[24,28–30] Recently, polymerase chain reaction (PCR)-based[31–37] and monoclonal antibody (MAb)-based[38–41] methods have been developed for quantitatively detecting salivary *S. mutans*. Our group had developed a set of MAbs,[41] which can quantitatively and accurately detect species-specific surface antigens of *S. mutans* in saliva and plaques.[39] The MAb-based *S. mutans* detection methods could provide a new opportunity for reexamining the relationship between salivary *S. mutans* counts and caries incidence. However, our previous investigation on application of these MAbs in quantitative detection of *S. mutans* was performed using defined bacterial species.[39,41] The accuracy of this method in detection and quantification of salivary *S. mutans* has not been systematically evaluated. This study aims to analyze the accuracy of the antibody-based detection method that we have developed previously in quantification of salivary *S. mutans* in comparison to commonly used selective media using PCR as the gold standard. The ultimate goal of the study is to identify a reliable way to quantitatively detect salivary *S. mutans*, which could potentially serve as an index for caries risk assessment.

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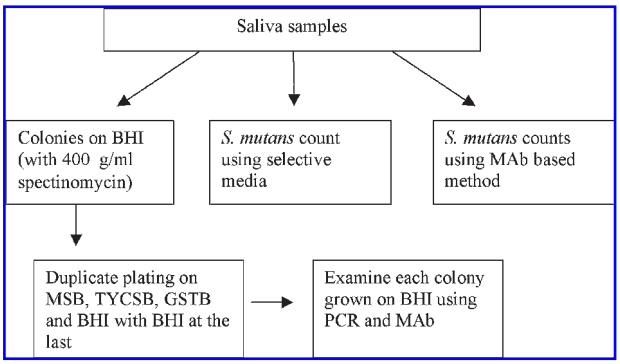

FIG. 1. Research design for this study.

MATERIALS AND METHODS

Research design

The research design for comparative analysis was shown in Figure 1. Saliva from each sample was diluted into appropriate concentrations and divided into three parts. One part was cultured on BHI with spectinomycin (Spc) plates (Spc was used to eliminate sensitive bacteria from saliva to ease colony counting) to count the total colony forming units (CFUs), which would include *S. mutans* and other species that are resistant to Spc and can grow on BHI plates. The second part was cultured on selective plates (MSB, TYCSB, GSTB) to select for *S. mutans*. The third part was used for counting *S. mutans* directly under the microscope using MAbs. After colonies appeared on BHI plates, they were serially transferred onto MSB, TYCSB, GSTB, and BHI plates with BHI plates last. The plates were incubated at 37°C anaerobically for 3 days. Colonies grown on the selective plates were counted, and colonies grown on BHI plates were used for colony PCR to identify *S. mutans* as well as stained directly with anti-*S. mutans* MAb. Data collected were used for statistical analysis.

Saliva collection

One milliliter of stimulated saliva samples was collected by asking participating human subjects to expectorate into sterile disposable plastic cups after the subjects chewed a piece of paraffin wax for 30 seconds. The saliva samples were immediately processed for microbiologic analysis.

Bacterial strains, media, and culture conditions

Streptococcal species *S. mutans* (ATCC 25175), *S. rattus* (ATCC 19645), *S. sanguis* (ATCC 19295), *S. sobrinus* (ATCC 33478), and *S. gondonii* (ATCC 10558), *Actinomyces naeslundii* (ATCC 12104), Lactobacilli *L. casei* (ATCC 11578), *L. acidophilus* (ATCC 4356), *L. plantarum* (ATCC 14917), and *L. salivarius* (ATCC 11742), and *Porphyromonas gingivalis* (ATCC 33277) were grown in brain–heart infusion (BHI, Difco 0037-17, Difco Laboratories, Detroit, MI) medium anaerobically (80% N_2, 10% CO_2, and 10% H_2) at 37°C. *Escherichia coli* was grown in Luria-Bertani (LB) medium with aeration or on LB agar plates at 37°C.

BHI plate and three commonly used selective media plates, MS Agar (Difco) with bacitracin (MSB), TYCSB, and GSTB, were prepared according to manufacturer's instructions and papers published previously.[26,27] To enrich streptococcal species from whole saliva samples, spectinomycin was added to BHI plates to a final concentration of 400 μg/mL after sterilization. All plates were stored in plastic bags at 4°C and used for a period of up to 1 month.

Enumeration of CFUs of saliva on culture plates

Stimulated saliva samples were diluted to appropriate concentrations and 100 μL of the dilutions of the samples were plated on BHI with Spc (400 μg/mL) or selective plates (MSB, TYCSB, GSTB). The plates were incubated at 37°C anaerobically for 3 days and colonies were counted. The assignment of colonies on the selective plates to *S. mutans* was done by colony morphology.

Enumeration of S. mutans *using fluorescent MAb*

Ten microliters of collected saliva sample or bacterial solution were mixed with 10 μL of culture supernatant of a hybridoma cell line producing an anti-*S. mutans* MAb, SWLA (about 10 μg antibody per milliliter),[39,41] and incubated at room temperature for 30 minutes. One microliter of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (IgG) was mixed with the saliva-MAb mixture and incubated at room temperature for 30 minutes. The mixture was then examined using fluorescent microscopy.[39] The number of fluorescent cells within six random observational microscopic fields was counted to reduce the variation. The corresponding colony forming unit per milliliter (CFU/mL) was calculated using a standard curve.

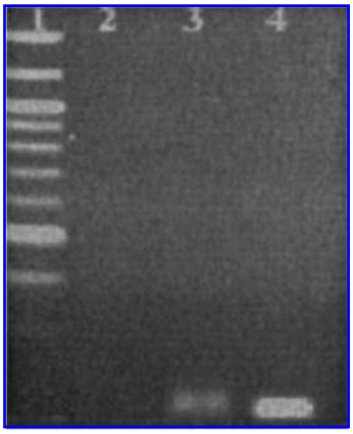

FIG. 2. The specificity of *Streptococcus mutans*-specific primers. Picture of gel electrophoresis of product from polymerase chain reaction (PCR) using the condition described in Materials and Methods. Lane 1 is DNA ladder. Lane 2 is the product from PCR using the mixture of DNA extracted from *S. rattus*, *S. sanguis*, *S. critatus*, *S. sobrinus*, *S. oralis*, *S. gondonii*, *Actinomyces naeslundii*, *A. viscosus*, *Lactobacilli casei*, *L. acidophilus* as the template. Lane 3 is the product from PCR using the same bacteria mixture and DNA of *S. mutans* as the template. Lane 4 is product from PCR using DNA of *S. mutans* only as the template. The specific PCR product is 134 bp.

Table 1. Number of *Streptococcus mutans* in Fifteen Saliva Samples Detected by Different Methods

	Salivary S. mutans *concentration (10^5/mL)*			
Sample no.	*MAb*	*MSB*	*TYCSB*	*GSTB*
1	3	0.4	0.5	0.2
2	12	22	4.3	0.1
3	42	26	10	0.1
4	26	0.2	0.4	0.1
5	12	0.1	20	0
6	10	20	19	0.1
7	12	20	20	0.2
8	8	3	4	4
9	7	8	5	1
10	9	4	6	5
11	21	4	15	10
12	15	7	12	8
13	16	7	12	10
14	8	5	7	6
15	22	4	10	14
Mean	14.87	8.71	9.68	3.92

MAb, monoclonal antibody; MSB, mitis-salvarius-bacitracin agar; TYCSB, trypticase yeast-extract cystine sucrose bacitracin agar; GSTB, glucose-sucrose-potassium tellurite bacitracin agar.

The standard curve was established as follows: overnight culture of *S. mutans* was ten-fold serially diluted, and 100 μL of the various dilutions were plated on BHI plates. The plates were incubated for 3 days under anaerobic conditions and colonies were counted, which was converted to CFU/mL. Concurrently, 10 μL of cell suspension from each dilution was labeled with MAb and the number of fluorescent cells counted as described above. The standard curve was generated using regression analysis with the number of fluorescent bacteria as the dependent variable and the logarithmic CFU as independent variables. The standard curve was created each time of sample processing to control random variations.

DNA extraction and PCR

DNA was extracted by mechanical disruption using a homogenizer (Omni International, Inc., Marietta, GA) for 30 seconds, and further purification by phenol-chloroform extraction. The purified DNA of each sample was ethanol precipitated and resuspended in 30 μL TE buffer (Tris-ethylenediaminetetraacetic acid [EDTA], pH 7). For PCR, DNA was diluted to a suitable concentration and 1 μL was used for each PCR

Table 2. Correlations Between Different Detection Methods for Salivary *Streptococcus mutans*

Detection methods	*MAb*	*MSB*	*TYCSB*	*GSTB*
MAb	1			
MSB	0.34			
	($p = 0.21$)	1		
TTCSB	0.09	0.31		
	($p = 0.76$)	($p = 0.26$)	1	
GSTB	0.11	−0.37	0.09	
	($p = 0.71$)	($p = 0.18$)	($p = 0.761$)	1

MAb, monoclonal antibody; MSB, mitis-salvarius-bacitracin agar; TYCSB, trypticase yeast-extract cystine sucrose bacitracin agar; GSTB, glucose-sucrose-potassium tellurite bacitracin agar.

Table 3. List of Data from Comparative Analysis Using MAb Selective Media

		Number of colonies			
Sample no.	*Test results*	*MAb*	*MSB*	*TYCSB*	*GSTB*
1	TP[a]	1	0	0	0
	TN[b]	73	65	64	64
	FN[c]	0	1	1	1
	FP[d]	0	8	9	9
2	TP[a]	4	1	1	1
	TN[b]	114	103	110	111
	FN[c]	0	3	3	3
	FP[d]	0	11	4	3
3	TP[a]	4	2	0	0
	TN[b]	177	119	174	177
	FN[c]	0	2	4	4
	FP[d]	0	58	3	0
4	TP[a]	29	21	23	20
	TN[b]	160	55	59	80
	FN[c]	4	11	13	13
	FP[d]	2	108	100	82
5	TP[a]	15	15	4	3
	TN[b]	11	7	9	12
	FN[c]	2	2	13	14
	FP[d]	2	6	4	1
6	TP[a]	44	53	47	53
	TN[b]	55	3	17	2
	FN[c]	4	0	6	0
	FP[d]	6	58	44	59
7	TP[a]	6	5	0	6
	TN[b]	44	6	35	0
	FN[c]	1	2	7	1
	FP[d]	0	38	10	44
8	TP[a]	4	1	0	0
	TN[b]	68	67	74	72
	FN[c]	1	5	5	5
	FP[d]	6	6	0	2
9	TP[a]	63	42	55	22
	TN[b]	114	79	86	82
	FN[c]	6	27	34	70
	FP[d]	5	40	13	1

[a]TP (true-positive) is defined as colonies grown on BHI plate that are PCR positive with *Streptococcus mutans*-specific primers as well as positive with MAb or other culture methods.

[b]TN (true-negative) is defined as colonies grown on BHI plate that are PCR negative with *S. mutans*-specific primers as well as negative with MAb or other culture methods.

[c]FN (false-negative) is defined as colonies grown on BHI plate that are PCR positive with *S. mutans*-specific primers but negative with MAb or other culture methods.

[d]FP (false-positive) is defined as colonies grown on BHI plate that are PCR negative with *S. mutans*-specific primers but positive with MAb or other culture methods.

MAb, monoclonal antibody; MSB, mitis-salvarius-bacitracin agar; TYCSB, trypticase yeast-extract cystine sucrose bacitracin agar; GSTB, glucose-sucrose-potassium tellurite bacitracin agar; PCR, polymerase chain reaction.

reaction. Two sets of primers were used for PCR. BA968F/BA1401R (5′-AACGCGAAGAACCTTAC-3′/5′CGGTGTGTACAAGACCC-3′)[42] amplifies 16S DNA of all eubacteria, and SmF205 (5′-ATTCCCTACTGCTGCCTCCC-3′) and SmR336 (5′-ATTCCCTACTGCTGCCTCCC-3′) amplifies only *S. mutans* 16S rDNA. The PCR mix (50 μL) contained 0.1 to 1 ng of template DNA, 1 mM $MgCl_2$, 5% (vol/vol) dimethyl sulfoxide (DMSO), 0.1 mM concentrations of each deoxynucleoside triphosphate, 0.4 μM concentrations of each primer, 1 U of *Taq* Polymerase, and TAQ buffer (Promega, Madison, WI), with final concentrations of 10 mM Tris-HCl (pH 8.3) and 10 mM KCl. Amplification conditions were initial denaturation at 94°C for 4 minutes, 30 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds, and final extension at 72°C for 7 minutes. For colony PCR, a single colony was used as template. The PCR mixture was treated at 95°C for 10 minutes before the application of the above amplification conditions.

Statistical analysis

The number of *S. mutans* was considered as continuous variable that follows normal distribution. Comparisons of the number of *S. mutans* detected with different methods were done with two-way analysis of variance (ANOVA). Pearson correlation analysis was also performed. Comparisons of the sensitivity, specificity, false-negative rate, and false-positive rate were done with one-way ANOVA. For multigroup comparisons, Bonferroni test was applied. Data analysis was performed using version 9.02 of SAS software (SAS Institute, Cary, NC). All statistical tests were two-tailed with a type I error of 0.05.

RESULTS

Number of S. mutans *in saliva samples detected with different methods*

The number of *S. mutans* from 15 saliva samples was obtained using MAb-based detection method and selective media, MSB, TYCSB, and GSTB, as described in Materials and Methods. The results are presented in Table 1. The mean of salivary *S. mutans* counts for these 15 subjects was in the order of MAb>TYCSB>MSB>GSTB, although the order varies for individual samples. Statistical analysis was performed using Proc ANOVA procedure in SAS (version 9.02). The results showed that there are statistically significant differences in the numbers of salivary *S. mutans* obtained with different methods ($p = 0.001$). The multigroup comparison analysis using Bonferroni test showed that the number of *S. mutans* obtained by MAb-based detection method is statistically significantly higher than the one provided by the GSTB method. The correlations in the numbers of salivary *S. mutans* between different detection methods were analyzed using Proc Corr procedure in SAS (version 9.02) and the correlation coefficiencies along with the associated *p* value are listed in Table 2. There are no statistically significant correlations between the different methods, suggesting that data obtained in different studies using different *S. mutans* detection methods are not comparable.

Comparative analysis of different detection methods

As shown above, there is no significant correlation in the number of salivary *S. mutans* between different detection methods. This suggests that some *S. mutans* may not grow on the selective media, while colonies that grew on selective media may not be *S. mutans*. To compare these methods further, we used PCR to test the *S. mutans* specificity of each method. Saliva samples from 9 of the 15 subjects were used in this analysis. As described in Materials and Methods, two sets of primers were used, one set detects *S. mutans* only, and the other set detects all bacteria. As shown in the experimental design (Fig. 1), each colony grown on a BHI plate was examined by PCR as well as MAb. To test the specificity of the *S. mutans*-specific primer, DNA was extracted from *S. rattus*, *S. sanguis*, *S. sobrinus*, *S. gondonii*, *Actinomyces naeslundii*, *Lactobacillus casei*, *L. acidophilus*, *L. plantarum*, *L. salivarius*, *Porphyromonas gingivalis*, and *E. coli* as described in Materials and Methods. A mixture of DNA extracted from the above organisms was used as template with or without the addition of *S. mutans* DNA. Only samples with *S. mutans* DNA were amplified, indicating that the *S. mutans*-specific primers are indeed species-specific (Fig. 2).

The specificity of this MAb for *S. mutans* has been reported previously.[39,41] Every colony on BHI plate was examined with MAbs. In cases in which the colony appeared to be a mixture of bacteria, the colony was considered as *S. mutans* positive if more than 50% of bacterial cells were labeled with the anti-

Table 4. Summary of Diagnostic Value of Different Methods

Method	*Sensitivity*[a]	*Specificity*[b]	*False-negative rate*[c]	*False-positive rate*[d]
MAb	0.91 ± 0.08	0.96 ± 0.06	0.07 ± 0.08	0.04 ± 0.06
MSB	0.53 ± 0.33	0.57 ± 0.33	0.48 ± 0.32	0.43 ± 0.33
TYCSB	0.29 ± 0.34	0.76 ± 0.27	0.70 ± 0.37	0.24 ± 0.27
GSTB	0.35 ± 0.38	0.68 ± 0.41	0.73 ± 0.31	0.32 ± 0.41

[a]Sensitivity is determined as TP/TP+FN.
[b]Specificity is determined as TN/TN+FP.
[c]False negative rate is calculated as FN/TP+FN.
[d]False positive rate is calculated as FP/FP+TN.
MAb, monoclonal antibody; MSB, mitis-salvarius-bacitracin agar; TYCSB, trypticase yeast-extract cystine sucrose bacitracin agar; GSTB, glucose-sucrose-potassium tellurite bacitracin agar; TP, true-positive; FN, false-negative; TN, true-negative; FP, false-positive.

body. The results of this comparative analysis are listed in Table 3. The calculations of sensitivity, specificity, false-positive rate, and false-negative rate of these methods are illustrated in Table 4. As shown in Tables 3 and 4, MAb-based detection method shows the highest sensitivity (90%), followed by selective medium MSB (53%), GSTB (35%), and TYCSB (29%). The MAb-based detection method also shows the highest specificity (96%) followed by TYCBS (76%), GSTB (68%), and MSB (57%). The differences in the sensitivities and specificities of these detection methods were analyzed using Proc ANOVA procedure in statistical software, SAS (version 9.02). There is a statistically significant difference in sensitivities between different methods ($p = 0.0007$). There are also statistically significant differences in false-negative ($p = 0.0001$) and false-positive rates ($p = 0.0001$). Unexpectedly, the differences in specificity between different methods are not statistically significantly different ($p = 0.057$), probably due to large variations and small sample size.

DISCUSSION

Dental caries is a prevalent chronic infectious disease. Despite the established role of *S. mutans* as the primary pathogen for dental caries, the diagnosis of dental caries is still mainly through nonmicrobiologic methods.[43,44] Previous studies on the association between the levels or proportions of *S. mutans* in saliva or dental plaque and the incidence of dental caries used conventional culture methods with different selective media to detect *S. mutans*.[6–23] These studies resulted in inconsistent, sometimes confusing, and conflicting results. To clarify some of the confusing issues and find a more accurate way for *S. mutans* detection, in this study we systematically compared the MAb-based method that we have established previously with three commonly used selective media based assays. We show that the different selective media as well as MAb-based methods have no correlation with each other in *S. mutans* detection, which may partially explain why conflicting results were obtained in previous studies from different groups.

Using PCR as the gold standard, we compared the accuracy of our MAb-based detection method and commonly used selective media for quantification of salivary *S. mutans*. Sensitivity, specificity, false-positive, and false-negative rates were calculated for each method. Overall, the MAb-based detection method showed significantly higher sensitivity, lower false-positive rate, and lower false-negative rate than conventional selective culture media (Tables 1–4). Although the specificity between different methods is not statistically different due to large variations in the data set of the selective culture methods, in reality, the MAb-based method is highly likely to have higher specificity than the culture methods, as demonstrated by its much lower false-positive rate. It is also worth noting that compared to the culture-based methods, the MAb-based method gives much smaller standard deviation in all four data sets (specificity, sensitivity, false-positive rate, and false-negative rate). This suggests that culture-based methods are more sensitive to variations in physiologic properties between different strains, while the MAb-based method is more robust probably due to its binding to a common surface antigen present in most, if not all, *S. mutans* strains. This may also explain the significantly higher number of *S. mutans* detected with the MAb methods than with selective culture methods from the same saliva samples (Table 1).

In addition to being more accurate, MAb-based detection is faster than selective culture assay. It requires only minutes while culture methods take days to see the results. Furthermore, samples for MAb-based analysis can be fixed, making transportation of samples more flexible, while culture based methods require live samples, which limits sample collection and processing only to microbiologic laboratories. These features make the MAb-based *S. mutans* detection a promising vehicle for future dental caries diagnosis. The drawback with the MAb-based detection technique is the cost of producing MAbs. However, with the advancement of MAb techniques, MAbs can now be produced in large quantity at relative low cost.

There are some limitations of this study. As mentioned in the Results section, the sample size was small, which generated large variations especially with the selective culture methods. Furthermore, in order to detect low numbers of *S. mutans* from saliva, a BHI plate with 400 μg/mL of spectinomycin was used. Previous studies have shown that *S. mutans* and other streptococci are resistant to as much as 500 μg/mL of spectinomycin (Qi et al., unpublished data). Although accurate estimates of *S. mutans* concentration (CFU/mL) can be obtained under this condition, the portion of *S. mutans* relative to other species present in saliva is overestimated. Nonetheless, since the number of *S. mutans* is what the tested methods were designed to detect, the proportion of *S. mutans* in the saliva population becomes irrelevant.

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