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Practical Method to Facilitate Estimation of *Streptococcus mutans* Levels in Saliva

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A method was developed to facilitate the estimation of *Streptococcus mutans* levels in saliva. Saliva-contaminated wooden spatulas were pressed directly against an elevated agar plate containing a selective medium. The results were compared with the number of *S. mutans* per 1 ml of paraffin-stimulated saliva. It was shown that the spatula method gave a good estimation of the level of *S. mutans* infection. The incubation was also made in expired air instead of 95% N_2–5% CO_2. The outgrowth was in good agreement with that after conventional incubation. The method is useful in epidemiological studies or in selecting persons at a high caries risk, and when ordinary saliva sampling cannot be done, for example in small children. Compared with conventional saliva sampling, this method requires less time and material at sampling as well as at the laboratory.

Several studies have reported a positive correlation between dental caries and the degree of infection with *Streptococcus mutans* (4, 9, 10). There is also some evidence that an increased level of *S. mutans* may precede the development of caries lesions (6, 9). Therefore, some interest has been generated in the feasibility of including this microorganism in caries prediction tests. Recent data also suggest that analysis of the number of *S. mutans* in saliva is superior in the prediction of caries than *Lactobacillus* counts, amount of dental plaque, saliva secretion rate, and salivary buffering capacity (7), at least in children of approximately 10 years of age.

As pointed out by Westergren and Krasse (13), current microbiological knowledge is rarely applied in general dental practice. This situation may be due in part to the reason that simple methods for bacteriological diagnosis are lacking. For example, analyses of *S. mutans* usually involve several steps such as collecting a saliva or a dental plaque sample, transferring it to a transport medium, and transporting it to a laboratory for processing, including dilution, plating, and the anaerobic incubation of several agar plates for each sample.

Although a recently published method involving the use of a micropipette (13) has simplified the procedure substantially, there is still a need for further improvements. In this paper, we present a method by which several of the steps mentioned above can be excluded. In addition, the method can be used for small children from whom saliva can be collected only with great difficulty. It is based on our earlier observation (8) that persons with different degrees of *S. mutans* infection transfer a proportional number of this organism when they contaminate a spoon with saliva.

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MATERIALS AND METHODS

The method involves briefly the following sequence of steps: (i) the sample is collected by placing a wooden spatula in the mouth in order to wet it with saliva; (ii) the spatula is pressed directly against a selective agar plate; (iii) plates are incubated anaerobically or in sealed plastic bags containing expired air; (iv) the number of bacterial colonies on a predetermined surface of the plates is estimated.

In this paper the results of the method have been compared with the results of conventional paraffin-stimulated saliva samples. The spatula method was performed twice: immediately before and after the paraffin chewing.

A first series of experiments included 37 adult patients. Sampling of bacteria from the oral cavity was performed with a 1.8-mm-wide wooden spatula (Fig. 1). About 3 cm of the spatula was introduced into the mouth and turned around 10 times to contaminate it with saliva. When the spatula was taken out of the mouth, any excess of saliva was wiped off against the lips. Each side of the spatula was then pressed against disposable contact petri dish (Nunc, Roskilde, Denmark; similar dishes are named RODAC plates in the U.S.) with an elevated level of the selective agar MSB (mitis salivarius agar with 15% sucrose and 3.3 mg of bacitracin, selective for *S. mutans*, per liter) (5) (Fig. 1). The agar plates were incubated at 37°C for 48 h in 95% N_2–5% CO_2.

The paraffin-stimulated saliva sample was then collected. Each subject chewed a piece of paraffin wax

(~2 g) for about 2 min. Saliva was collected at the same time, and 1 ml of the sample was then transferred to 1 ml of reduced transport fluid consisting of a balanced mineral salt solution poised with dithiothreitol and containing ethylenediaminetetraacetic acid (RTF) (12) and brought to the laboratory for preparation within 3 h. The number of *S. mutans* colony-forming units (CFU) per 1 ml of saliva was estimated by the micropipette method described by Westergren and Krasse (13).

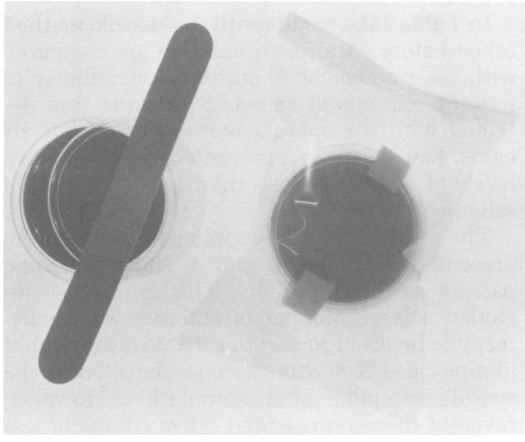

FIG. 1. *MSB agar dish with an elevated level of agar and a wooden spatula (left). Sealed plastic bag containing a MSB agar dish in expired air (right).*

Immediately after the paraffin-stimulated saliva sample was collected, the sampling with the spatula method was repeated. The plates were incubated as mentioned above, and the number of colonies resembling *S. mutans* on a predetermined area of the tip (approximately 1.5 cm^2) were counted for each side pressed against the MSB agar (Fig. 2). The mean of the two observations for each sample was compared with the results of the conventional saliva samples. To obtain an estimation of the reproducibility of the results, the number of colonies on one side of the spatula was compared with the number on the other side. In this comparison, samples of 115 subjects were included.

The spatula method, with a few modifications, was also used for a group of children, 3 to 6 years old, attending a public dental clinic in Göteborg. Instead of turning the spatula around, the children sucked a couple of times on each side of the spatula until it was

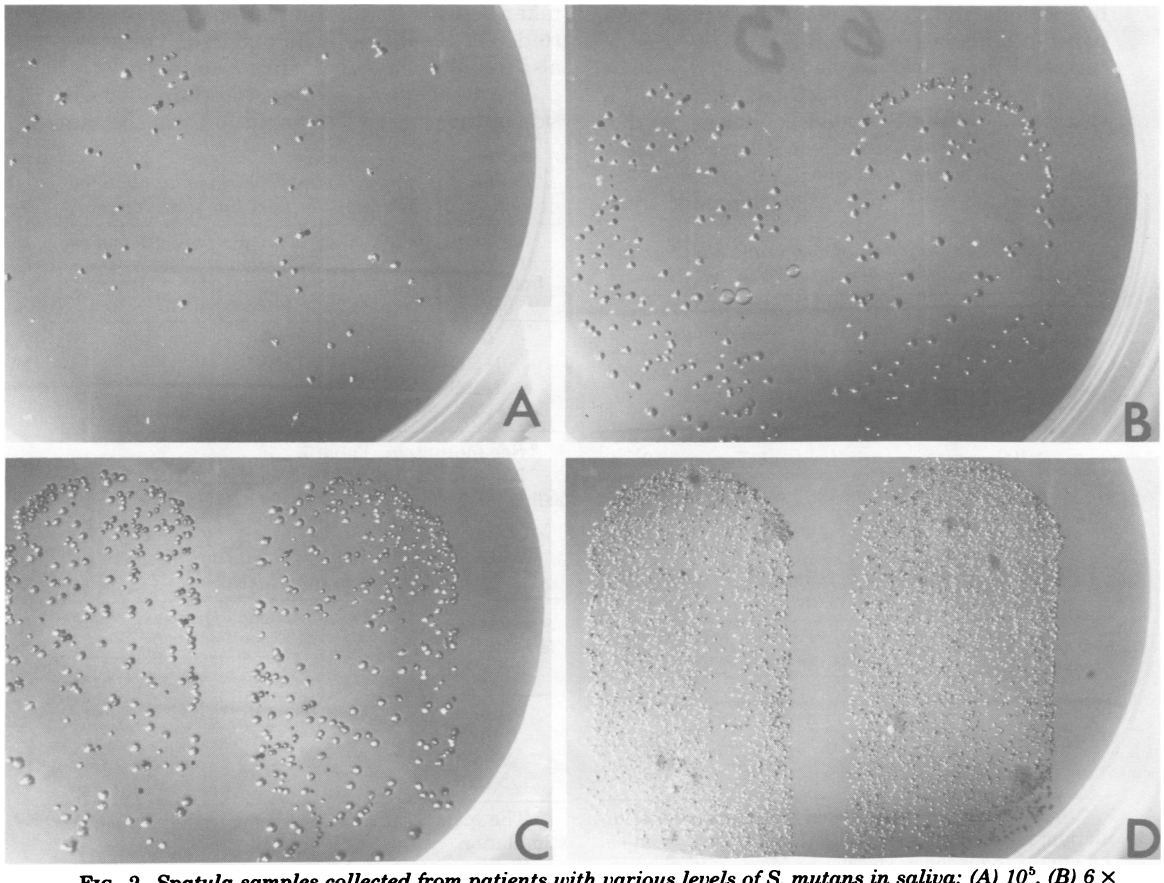

FIG. 2. *Spatula samples collected from patients with various levels of S. mutans in saliva: (A) 10^5, (B) 6×10^5, (C) 10^6, (D) 9×10^6 S. mutans per ml of saliva.*

moistened. For stimulation, a small piece of paraffin wax (~0.7 g) was then used. Depending on the child's ability to cooperate, 0.1 to 0.5 ml of paraffin-stimulated saliva was collected. Again, the spatula method was used both before and after the paraffin chewing. The preparations of the samples were performed as mentioned above. Eighty-six children participated in this part of the study.

To find out if *S. mutans* could be cultivated even more simply by the elimination of anaerobic jars and gas tanks used for routine culture procedures, saliva-contaminated MSB agar plates were placed in plastic bags (Plasteril folie, Plate Bonn GMBH, Bonn, Germany) (Fig. 1). The opening of the bag was then almost closed by welding (Impulse-welding apparatus, Elwis, Gentofte, Denmark). The bag was then filled with expired air from one staff member and immediately sealed completely. The bag was placed at 37°C for 48 h. As a control, duplicate samples collected at the same time were incubated in the conventional way. This study included samples from 54 subjects.

RESULTS

In Tables 1 and 2 the results from the sampling of adults are presented. Table 1 shows the comparison between the paraffin-stimulated saliva sample and the spatula sample taken after paraffin stimulation. When *S. mutans* was detected in the saliva sample it was also detected by the spatula method, with one exception. In this case, however, the saliva count was less than 10,000 *S. mutans* per ml of saliva. Increased numbers of *S. mutans* per 1 ml of saliva also corresponded with increased numbers transferred by the spatula. Thus when the saliva counts were more than 10^6 *S. mutans*, the number transferred by the spatula was more than 100 CFU.

TABLE 1. *Comparison of S. mutans numbers in saliva of adults after paraffin stimulation*

S. mutans transferred from spatula[a] (CFU)	No. of saliva samples having *S. mutans* (CFU/ml) numbers:		
	$0–10^5$	$10^5–10^6$	$>10^6$
0–20	9	4	
21–100		11	
>100		1	12

[a] Counts transferred from ca. 1.5 cm^2 of the wooden spatula to MSB agar.

TABLE 2. *Comparison of S. mutans numbers in saliva of adults*[a]

S. mutans transferred from spatula (CFU)	No. of saliva samples having *S. mutans* (CFU/ml) numbers:		
	$0–10^5$	$10^5–10^6$	$>10^6$
0–20	9	11	2
21–100		5	6
>100			4

[a] Spatula samples were taken before paraffin stimulation; numbers represent CFU transferred from ca. 1.5 cm^2 of the wooden spatula to MSB agar. Conventional saliva samples were taken after paraffin stimulation.

In Table 2 the results with the spatula method taken before paraffin stimulation are compared with the number of *S. mutans* per milliliter of paraffin-stimulated saliva. *S. mutans* was detected with the spatula method in all but six cases. Four of these represented very low saliva levels of *S. mutans* (less than 4×10^4 per ml of saliva).

The results from the sampling of the children, presented in Tables 3 and 4, show the same pattern as for the adults. This group also included a large number of children with undetectable levels of *S. mutans*. Table 3 shows that in nine cases *S. mutans* was not detected by the spatula sampling after stimulation. However, seven of these represented saliva counts of less than 10,000 *S. mutans* per ml of saliva. On the other hand, in two cases low numbers of *S. mutans* were detected by the spatula method but not in the saliva sample. The spatula sample taken before paraffin stimulation (Table 4) failed to detect *S. mutans* in 15 children. These were also found to have low saliva counts.

The number of *S. mutans* transferred by each side of the spatula was found to be in the same

TABLE 3. *Comparison of S. mutans numbers in saliva of children after paraffin stimulation.*

S. mutans transferred from spatula[a] (CFU)	No. of saliva samples having *S. mutans* (CFU/ml) numbers:			
	0[b]	$1–10^5$	$10^5–10^6$	$>10^6$
0	40	9		
1–20	2	13		
21–100		2	6	
>100			3	11

[a] See footnote *a*, Table 1.

[b] No colonies (0) means less than 400 CFU/ml of saliva.

TABLE 4. *Comparison of S. mutans numbers in saliva of children*[a]

S. mutans transferred from spatula[b] (CFU)	No. of saliva samples having *S. mutans* (CFU/ml) numbers:			
	0[c]	$1–10^5$	$10^5–10^6$	$>10^6$
0	40	15		
1–20	2	8	7	5
21–100		1	1	4
>100			1	2

[a] The spatula samples were collected before paraffin stimulation.

[b] See footnote *a*, Table 1.

[c] No colonies (0) means less than 400 CFU/ml of saliva.

magnitude in 84% of the samples. In 15% of the samples the difference was one group of magnitude.

The numbers of *S. mutans* CFU recovered by the two incubation methods are compared in Table 5. In 85% of the samples the numbers found by the two methods were within the same range. The conventional way of incubation gave a higher number of *S. mutans* in only 9% of the samples.

DISCUSSION

The numbers of *S. mutans* colonies obtained with the spatula method after paraffin chewing were closely related to the numbers of CFU in the stimulated saliva samples (Tables 1 and 3). This indicates that the new method reflects the salivary levels of *S. mutans*. Saliva samples instead of dental plaque samples have sometimes been used for estimation of the degree of infection with *S. mutans*. The reason for this is the localized way in which *S. mutans* colonizes the teeth (1, 3, 11). Plaque samples from different teeth may thus show very large variations in their contents of bacteria. High numbers of *S. mutans* in a saliva sample, on the other hand, indicate either that several teeth are infected or that fewer surfaces are more heavily infected. In each case, however, the subjects themselves can be regarded as heavily infected.

Paraffin chewing increases the shedding of bacteria from the teeth. A comparison of the spatula method performed before chewing with a paraffin-stimulated saliva sample is therefore not quite correct. It was included in the present study to see whether the spatula method could be used also for patients who cannot chew paraffin readily, for example very young children. Although an unstimulated sample seems to be useful for the determination if an infection is present or not, it does not always reflect the release of bacteria at chewing.

TABLE 5. *Comparison of S. mutans numbers detected after anaerobic and expired-air incubation*[a]

S. mutans CFU[b]: expired air	No.[b] of anaerobically incubated samples having *S. mutans* numbers:		
	0–20	21–100	>100
0–20	20	2	
21–100	2	13	3
>100		1	13

[a] Anaerobic incubation was in jars filled with 95% N_2–5% CO_2; expired-air incubation was in sealed plastic bags filled with expired air. 95% confidence limits for the mean difference between the two culturing methods: 2 ± 6.

[b] The mean of the number of colonies transferred by the two sides of the spatula.

We have found the spatula method very simple to handle. It is fast, and no transport media or dilution steps are necessary. These facts make it convenient to use in epidemiological studies or in dental practice. To further minimize the equipment needed if samples are processed outside a microbiology laboratory, the experiments using incubation in an atmosphere of expired air instead of laboratory gas mixtures were performed. *S. mutans* recoveries were similar with the two methods. In contrast, control plates incubated aerobically without expired air showed no growth at all or considerably fewer colonies.

By using the spatula method, highly infected patients can be quickly detected by judging the density of the outgrowth (see Fig. 2). Patients with no or very low levels, below 10^4 CFU/ml, of *S. mutans* are also easily identified. The method thus seems to be sufficient for finding subjects who can act as possible sources for transmission of *S. mutans* infection. Considering the findings by Klock (7), it may also be used for identifying patients with increased risk for caries as well as for evaluation of caries-preventive measures. At the Department of Cariology in Göteborg, usually a level of 10^6 *S. mutans* or more per 1 ml of saliva is considered very high and justifies the use of intensive therapy to decrease the number. The lowest limit of detection with the method presented is at least 100 times below this high level.

The method described is based on the highly selective medium MSB. Some of its properties have been studied earlier at our laboratory (2). It can be mentioned here that the problem with outgrowth of species other than *S. mutans* is little, if the agar plates are used freshly prepared (maximum 1 week old). When outgrowth of other species occurs, such strains can usually be excluded by studying the colonial morphology under a dissecting microscope. Colonies with slightly aberrant forms can be detected, however. These colonies need further identification, but the number of patients with such colonies is so low that they should not detract from the advantages of the method.

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