A New Method for the Estimation of Mutans Streptococci in Human Saliva

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A new method for quantitating the numbers of mutans streptococci in saliva—the “Strip mutans” test—was developed. It includes the following steps: A prepared plastic spatula is contaminated with saliva, transferred to a selective broth, incubated for two days, and dried. A predetermined area is counted or evaluated for CFUs of mutans streptococci. The medium contains sucrose to promote adhesion of mutans streptococci to the strip. Bacitracin, added just before use, is the basis for the selectivity. Results with the new medium were compared with those from the “spatula technique” and with those from conventional saliva sampling with dilution. The studies involved 302 and 60 schoolchildren, respectively. There was a highly significant correlation between the results from the new method and those from conventional methods. The strip mutans test is easy to use and has long shelf-life, and the spatulas with adherent colonies can be saved for future comparisons.


Introduction.

This paper describes a new method for the quantitation of mutans streptococci in saliva. The background for our work is based on the following facts:

(1) Several papers indicate that mutans streptococci (S. mutans and S. sobrinus) are usually associated with dental caries (Emilson and Krasse, 1985; Loesche, 1986).

(2) Mutans streptococci colonize the teeth, and the salivary level reflects the number of colonized sites (Emilson et al., 1982; Tegelius et al., 1984).

(3) The number of mutans streptococci in saliva can be used for the evaluation of caries risk and is also useful for monitoring the level of colonization of the individual (Zickert et al., 1982; Krasse, 1984).

(4) Simple methods are a prerequisite for making microbial tests a routine in clinical work, and different methods, developed during the last few years (Matsukubo et al., 1981; Alaluusa et al., 1984; Jordan et al., 1987), are indications of that need. Although there are a number of methods available for the determination of mutans streptococci (Brathall and Carlsson, 1989, for review), we believe that further improvements are necessary to make them more clinically applicable.

Materials and methods.

The study included four parts:

(1) comparison between the number of colony-forming units (CFUs) of mutans streptococci obtained according to the new method—“Strip mutans”—and the number of CFUs growing on selective agar plates, according to the “spatula method” (Köhler and Brathall, 1979);

(2) estimation of the reliability of the new method. The numbers of CFUs on two plastic spatulas, contaminated immediately after each other and incubated separately, were counted;

(3) comparison between the mean CFUs from two plastic spatulas, with the counts of conventional plating of paraffin-stimulated saliva on MSB-agar plates; and

(4) comparison between the results of the “spatula method” and those of conventional plated saliva samples.

Subjects. — All pupils in a school in the city of Malmö, Sweden, were included. The ages varied from 7 to 12 years, with about the same number of children in each age group. A few weeks before the study, the children received a written description to be forwarded to their parents, explaining the purpose of the study. Only three children refused to participate, and one declined to give saliva, leaving 302 children in this study.

Collection of saliva. — The subjects chewed a piece of paraffin wax, about 0.9 g, for one minute. Then, a plastic strip (8 × 73 mm) (Orion Diagnostica, Helsinki, Finland), which had one side roughened to favor adherence, and a wooden spatula (18 × 150 mm) (Param Grosshandelsgesellschaft, Hamburg, FRG) was turned around 10 times in the mouth to be contaminated with saliva and bacteria. Any excess of saliva was removed by withdrawal of the spatula between closed lips. In half the number of classes, we started with the plastic spatula, immediately followed by the wooden spatula. In the other classes, wooden spatulas were used first.

In the 61 12-year-old children, we took two consecutive plastic spatula samples either before or after the wooden spatula, followed by a paraffin-stimulated saliva sample collected in a test tube. About 5 mL of saliva was obtained from each child.

Incubation and counting. — The plastic spatulas were immediately transferred to test tubes with 6 mL of a selective broth. The buffer base was similar to that used in MSB agar (Gold et al., 1973). To promote adhesion of colonies to the strip, we increased the sucrose concentration to 30%. The bacitracin solution, to select for mutans streptococci, had been prepared separately and an aliquot (30 µg) dried on small filter papers which were added to the broth prior to use, giving a final concentration of 0.36 U of bacitracin per mL. The tubes were filled with expired air and closed with screw caps.

The wooden spatulas were pressed directly against the elevated surfaces of MSB agar in contact petri dishes (Nunc, Roskilde, Denmark), according to the original spatula method described by Köhler and Brathall (1979). The plates were incubated in plastic bags containing expired air.

One mL of the saliva samples was immediately transferred to VMG transport medium (Möller, 1966) and then plated on MSB-agar plates (Klock and Krasse, 1979) within eight hr and incubated in 95% N2, 5% CO2. All tubes, NUNC-plates, and regular agar plates were incubated at 37°C for 48 hr.

After incubation, the plastic spatulas were removed from the broth and dried at room temperature. The number of CFUs on a predetermined area of the strip, 10 × 8 mm, was counted under 10× magnification. Four levels were chosen for the results—0 CFU, 1–10, 11–99, and ≥100 CFUs—and the same groups were used for the spatula method. The conventional agar plates were counted, and the numbers of CFU per mL saliva grouped at four levels: < 104, 104–105, 105–106, and > 106.

The bacterial counts obtained by the different sampling
methods as well as the duplicate samples obtained by plastic spatulas were compared by frequency analysis using the contingency coefficient (Sharp, 1979), calculated as \( C = \frac{X^2}{(X^2 + N)}^{0.5} \), where \( N \) is the total number of subjects in the study. The comparisons were all based on 4 \( \times \) 4 groups where the maximal contingency coefficient is 0.866.

From the 46 12-year-old children with strips showing growth, 59 colonies, representing various morphological types, were isolated and subjected to immunofluorescent identification, using antisera reacting with serotypes c/e/f (S. mutans) and d/g (S. sobrinus), respectively (Brathall and Pettersson, 1976; Carlsson et al., 1985).

**Results.**

Table 1 shows the comparison between the number of CFUs on “Strip mutans” and on the agar plates according to the “spatula method” (wooden spatulas). The two samples from each subject coincided in 80% (241 cases), and 19% showed a discrepancy of one class. The Fig. shows some examples of the appearance of the colonies on the strips. When examined under a microscope, the colonies appeared similar to those found on MSB-agar plates.

Table 2 shows the reliability of two consecutive “Strip mutans” samples. 87% of the samples agreed, and the remaining did not differ by more than one class.

Table 3 shows the comparison between the results of “Strip mutans” and those from the collected saliva samples, processed according to conventional methods. The grouped results for the dilution-plate count method were in accordance with the counts on the strip in 77% of the subjects. A few, 16%, showed higher values for the spatula, and 7% lower. Only one sample differed by two classes.

Table 4 shows a similar comparison for the spatula method and the conventional plates. Similar results as for the strip were obtained.

Of the 59 colonies studied, five did not react with the antisera used.

**Discussion.**

This paper describes a new method for the enumeration of mutans streptococci in saliva. We believe that the method will be useful, both at the individual level—for example, when evaluating important caries risk factors in a single case or monitoring a patient undergoing preventive treatment—and on the population level, as one measure in attempts to identify groups of persons with increased risk to develop caries. To

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**TABLE 1**

<table>
<thead>
<tr>
<th>Strip mutans, CFU</th>
<th>Spatula method, CFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1-10</td>
<td>1</td>
</tr>
<tr>
<td>11-99</td>
<td>0</td>
</tr>
<tr>
<td>≥100</td>
<td>0</td>
</tr>
</tbody>
</table>

*Number of samples.
Total number of saliva samples = 302.
Contingency Coefficient = 0.78.

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**TABLE 2**

<table>
<thead>
<tr>
<th>Strip mutans, Sample 1, CFU</th>
<th>Strip mutans, Sample 2, CFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>11-99</td>
<td>0</td>
</tr>
<tr>
<td>≥100</td>
<td>0</td>
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</tbody>
</table>

*Number of samples.
Total number of saliva samples = 61.
Contingency Coefficient = 0.80.

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**TABLE 3**

<table>
<thead>
<tr>
<th>No. of saliva samples having mutans (CFUs/mL) counts</th>
<th>Strip mutans, CFU*</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;10^4</td>
<td>14* 3 0</td>
</tr>
<tr>
<td>10^3-10^4</td>
<td>0 0 3</td>
</tr>
<tr>
<td>10^5-10^6</td>
<td>1 0 13 4</td>
</tr>
<tr>
<td>&gt;10^6</td>
<td>0 0 3 19</td>
</tr>
</tbody>
</table>

*Mean number of two consecutive Strip mutans samples.
†Number of samples.
Total number of saliva samples = 60.
Contingency Coefficient = 0.76.

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**TABLE 4**

<table>
<thead>
<tr>
<th>No. of saliva samples having mutans (CFUs/mL) counts</th>
<th>Spatula method, CFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;10^4</td>
<td>14* 2 1 0</td>
</tr>
<tr>
<td>10^3-10^4</td>
<td>0 1 2 0</td>
</tr>
<tr>
<td>10^5-10^6</td>
<td>0 2 13 3</td>
</tr>
<tr>
<td>&gt;10^6</td>
<td>0 0 4 18</td>
</tr>
</tbody>
</table>

*Number of samples.
Total number of saliva samples = 60.
Contingency Coefficient = 0.75.
clarify the special features of the method, we would like to describe the background in more detail. Some years ago Köhler and Bratthall (1978) studied the circumstances by which *S. mutans* (mutans streptococci) could be transferred from mother to child. As part of that investigation, the number of *S. mutans* on a spoon, contaminated by saliva, was evaluated. It turned out that the more *S. mutans* the mother had in her saliva, the more were transferred to the spoon. These findings resulted in a "practical method to facilitate estimation of *S. mutans* levels in saliva" (Köhler and Bratthall, 1979), where the spoon was replaced by a flat wooden tongue blade. The method, described above, is often referred to as the "spatula method". That method has been used in several studies, some of them under field conditions (Carlsson et al., 1985; El Tayeb et al., 1985). A limitation of the method, however, is the short shelf-life, maximum of one week, since it involves MSB agar. This is particularly inconvenient when the plates are used in a clinical office or in remote areas. Many attempts to prolong the storage capacity of such agar plates have been undertaken in our laboratories, but in our judgment, no real improvement was obtained until we arrived at the present method.

Considerable efforts have also been made by others with the aim of obtaining practical methods for clinical use. Matsukubo et al. (1981) developed a method with long shelf-life, where saliva is transferred into a tube, activated by addition of selective ingredients on a strip just before use. The mutans-bacteria are growing on the glass, and the method is thus based on both selectivity of the broth and adherence of mutans to glass. The amount of bacteria on the glass is estimated as belonging to one of four different density groups. Alaluusa et al. (1984) presented a method which has a prolonged storage capacity if the discs containing bacitracin are added to agar slides after saliva has been poured over the surface. Bacitracin diffuses from the discs, and only bacteria resistant to bacitracin can grow close to the discs. Another more recent method with extended shelf-life has been described by Jordan et al. (1987). According to their method, saliva is collected in a vial containing a diluent to which bacitracin is added before use. A dipslide with agar is dipped into this mixture. Another vial is prepared in the meantime with a CO₂ tablet, and the slide is then transferred to this second vial and incubated.

In our method, the concentrations of bacitracin and sucrose were chosen to obtain distinct colonies, good adhesion to the strips, and increased preservation capacity of the colonies after being dried. In the interval of 1–10 CFU, the separate colonies were bigger than on strips with more dense growth. Such colonies have a somewhat reduced adhesion ability and may sometimes fall off when the strip is removed from the tube. It is therefore recommended that the strip be checked while it is still in the broth. Colonies were never found at the bottom of the tubes when more dense growth was obtained. It should also be pointed out that the mutans bacteria are growing on the strip, not in the medium. In rare cases when such growth may be found, it will consist of bacteria which can resist the sucrose/bacitracin combination but which do not, like mutans streptococci, have the ability to adhere. Growth of yeasts may be such an example. It appeared in three of our samples. In a few further cases, colonies with a morphology not resembling mutans were found on the strips. They were easily distinguished from mutans, but further work must be done to determine their identity. The use of expired air in the tubes may be a step in the procedure which can possibly be excluded. It was introduced because it is a step in the spatula method but may not be necessary with the present technique.

The particular plastic strip was chosen since it gave levels of the same magnitude of mutans streptococci as the "spatula method". The vast clinical experience acquired with the latter method can thus be used for the new method as well. The spatula method uses MSB-agar, a material which has been subjected to some criticism for suppressing the recovery of mutans streptococci (van Palenstein Helderman et al., 1983; Tanzer et al., 1984). Although such results cannot always be confirmed (Beighton, 1986), it should be pointed out that all selective media suppress the mutans streptococci to a certain extent. This fact is of course important but must be seen in relation to the clinical use. If a method is able to separate a population into groups with different caries development, then the method is useful even if the absolute numbers of bacteria may be underestimated. Actually, it may even be a disadvantage with a too-sensitive method, since subjects with very low levels of mutans streptococci will appear positive, with little clinical relevance. For MSB-agar, there are more studies indicating clinical usefulness than for any other medium described so far.

The main advantages with our new method are: (i) its simplicity—it can easily be handled by personnel not trained in laboratory work; (ii) its long shelf-life, since the bacitracin is added just before use, and it is based on broth instead of agar; (iii) the fact that the strips are easy to read, colonies can be counted, and the morphology can be checked; (iv) the fact that separate colonies can be isolated for further identification if necessary; (v) its suitability for chairside instructions, since the colonies are preserved on the strip after being dried, and the results can be saved a long time for further comparisons.

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REFERENCES


