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Enumeration of Mutans Streptococci in Clinical Samples by Using Monoclonal Antibodies

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Mutans streptococci have been strongly associated with dental caries. Two members of this group of bacteria, *Streptococcus mutans* and *Streptococcus sobrinus*, are often found in human dental plaque. Identification of mutans streptococci on the basis of sugar fermentation is troublesome and easily leads to erroneous conclusions. Furthermore, the recovery on selective media differs for different species. This causes incorrect enumeration of *S. mutans* and *S. sobrinus* in clinical samples. The aim of this study was to develop a method for simultaneous identification and enumeration of *S. mutans* and *S. sobrinus* in dental plaque and saliva samples. With this immunoblot technique (IBT), significantly more plaque samples containing *S. sobrinus* were detected than on the selective medium Trypticase-yeast-cysteine-sucrose-bacitracin agar (TYCSB) ($P < 0.01$). The numbers of plaque samples harboring *S. mutans* were equal on TYCSB and by IBT. However, the numbers of CFU of *S. mutans* as well as of *S. sobrinus* detected with the IBT were significantly higher than those obtained on TYCSB ($P < 0.001$). The recovery of primary isolations of *S. sobrinus* on TYCSB seems to have been inhibited in 26 of the 45 *S. sobrinus*-containing plaque samples. False-positive or false-negative reactions with the IBT were not found.

Mutans streptococci are strongly correlated with dental caries (19–21, 23). In a previous paper we reported on the higher acidogenic capacity of *Streptococcus sobrinus* compared with *Streptococcus mutans* (9). Others suggested a correlation between *S. sobrinus* and approximal caries (16, 17, 22). These results suggest that it is important to discriminate critically between the several species of the mutans streptococci.

Several methods have been developed to measure the number of mutans streptococci in dental plaque or in saliva (13, 28, 30, 34). These methods are based on the relative resistance of mutans streptococci to bacitracin, balancing between optimal inhibition of background bacteria and optimal growth of mutans streptococci. This means that often growth of the mutans streptococci is inhibited as well (31). It has been reported that *S. sobrinus* especially is difficult to culture directly from dental plaque on selective media and that up to 75% of these bacterial strains can be inhibited on mitis salivarius bacitracin (MSB) selective medium (18, 31, 35). These studies show that Trypticase-yeast-cysteine-sucrose-bacitracin agar (TYCSB) is the best selective medium on which to isolate and count *S. sobrinus* from dental plaque (28, 34).

In several epidemiological studies, identification of *S. mutans* and *S. sobrinus* on selective media has been performed by using colony morphology. We previously reported that on the basis of colony morphology, correct differentiation between *S. mutans* and *S. sobrinus* is not possible (10). Other identification methods, besides immunological methods, are based on sugar fermentation patterns (12, 25). In epidemiological studies, identification of several colonies with sugar fermentation is laborious and is therefore not often performed. We have, moreover, previously reported that differentiation between *S. mutans* and *S. sobrinus* is not possible with sugar fermentation patterns (10). In these studies, the guanine-plus-cytosine content of the DNA served as a standard. However, determination of the G+C content of the DNA is time-consuming and therefore not suitable for use in standard identification in epidemiological studies.

Immunological probes with polyclonal antibodies have been successfully used for many years for the serotyping of mutans streptococci (1–5, 24, 25, 32). These polyclonal antibodies are highly active against different antigenic determinants and give a strong positive signal. However, the disadvantage is that these antibodies may be cross-reactive against several other microorganisms. This cross-reactivity should be absorbed for each new batch of antiserum, and therefore, the characteristics of these antisera may differ from batch to batch.

The use of monoclonal antibodies (MAbs) has the advantage that this antiserum is checked for cross-reactivity once, after the isolation. These characteristics will remain as long as the corresponding hybridomas are available. We reported previously on the isolation of MAbs specific for *S. sobrinus*, *S. mutans*, and *Streptococcus cricetus* (10a).

The aim of this study was to develop a method to enumerate and identify mutans streptococci in saliva and in dental plaque samples. This paper describes an immunoblot technique (IBT) (which is a modified colony blot technique) in combination with culturing the specimens on a nonselective medium. The reliability of this method was tested with laboratory strains, with dental plaque samples from rats which were inoculated with *S. sobrinus* and *S. mutans*, and with plaque samples from Dutch volunteers.

MATERIALS AND METHODS

IBT. The MAbs used in this study were OMVU2, specific for mutans streptococci; OMVU10, specific for *S. sobrinus*; OMVU31, specific for *S. mutans*; and OMVU40, specific for *S. cricetus*. The characteristics of all MAbs are described elsewhere (10, 10a).

Dental plaque samples were sonicated 10 times for 1 s and

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diluted 1:100 in Todd-Hewitt broth (Oxoid Ltd., Basingstoke, Hants, England). This dilution was used to inoculate nitrocellulose membranes (pore size, 0.45 μm) (HA type; Millipore) with a spiral plater (spiral plating system; Lameris). These membranes were previously placed on blood agar plates (blood agar base no. 2 [Oxoid] supplemented with 5% sheep blood). The plates, still with the membranes on the top, were incubated for 2 days anaerobically at 37°C (80% N_2, 10% H_2, and 10% CO_2). Next, the membranes were peeled off, dried at 40°C for 30 min, and incubated with phosphate-buffered saline (pH 7.4) supplemented with 0.05% Tween 80–0.001% Merthiolate (PBST) and with 1% bovine serum albumin for 30 min at room temperature. To check for microorganisms which are (cross-)reactive with horseradish peroxidase-conjugated goat anti-mouse immunoglobulins (affinity purified, 170-6516; Bio-Rad, Richmond, Calif.), the membranes were incubated with this antiserum (diluted 1:1,500 in PBST) at room temperature for 2 h. After 5 5-min wash steps with PBST, a staining procedure with a chloronaphthol solution (50 mg of chloronaphthol in 20 ml of 96% ethanol, 80 ml of citrate [10 mM; pH 5.5], and 40 μl of 30% H_2O_2) was performed. The positive dots were marked on a transparent sheet. To identify *S. sobrinus* colonies, the membranes were washed once with PBST and incubated with MAb OMVU10 against *S. sobrinus* at room temperature for 16 h. After three wash steps with PBST, the adherence of the MAbs to the bacteria was made visible with a conjugated goat anti-mouse immunoglobulin incubation and by the staining procedure described above. After the newly stained dots, which correspond to *S. sobrinus* colonies grown on the nitrocellulose membrane, were marked on the same transparent sheet as used before, the incubations were continued with a MAb against *S. mutans*, OMVU31. The adherence of this MAb was detected by the same method as described for OMVU10. After the detection of these *S. mutans*-positive colonies, a final incubation step was performed with OMVU2. The membranes were treated for OMVU2-positive colonies in the same manner as for the OMVU10 and OMVU31 antibodies.

All incubation steps were performed in roller bottle flasks which were placed on a Rollo cell incubator (New Brunswick Scientific, Edison, N.J.) and rotated slowly (approximately 3 rpm).

Rat dental plaque samples. The strains used to inoculate the rats were all isolated from dental plaques of 9-year-old children, subcultured twice, and identified with MAbs as described elsewhere (10a). The strains used in this study were *S. mutans* HG979, HG982, HG983, HG1003, HG1010, and HG1012 and *S. sobrinus* HG961, HG962, HG970, HG977, and HG976.

Rats (22 days old) were inoculated, according to a standard procedure, with 11 different strains of *S. mutans* and *S. sobrinus* that had been grown for 16 h, 8 rats per strain. The rats were fed diet SSP 20/5, containing 20% sucrose and 5% glucose, ad libitum (15). After 35 days, the rats were killed and the maxillary molars were extracted and homogenized (15, 33). The bacteria in the homogenates were cultured and identified as described for the IBT. To compare the results with those of a selective medium, the homogenates of the rat molars were also inoculated on TYCSB (34). The total anaerobic viable counts were measured on blood agar plates without nitrocellulose membranes. All plates were incubated anaerobically (80% N_2, 10% CO_2, and 10% H_2) at 37°C. Blood agar plates were incubated for 2 days, whereas the TYCSB plates were incubated for 4 days. Dominant mutans streptococci were isolated from each plaque sample and identified by comparing cell wall protein patterns of these strains with those of reference strains by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (26, 27).

Human dental plaque samples. Dental plaque was isolated from 51 volunteers (medical students, aged 20 to 25 years) by scraping with a toothpick between the first and second molars and was processed as described above for the IBT. TYCSB plates were inoculated by using the same dilution as was used for the IBT and were incubated anaerobically at 37°C for 4 days. The numbers of CFU of *S. mutans* and *S. sobrinus* on TYCSB were determined by the method of van Palenstein Helderman et al. (34), which is based on the presence of a white precipitate around the colonies. To determine whether all mutans streptococcal colonies were correctly identified and no colonies were missed, replicates were made of each nitrocellulose membrane. This was performed by gently pressing the side of the membranes on which the colonies were grown on blood agar plates and on TYCSB plates just before the IBT staining procedure was started. All colonies which reacted with one of the MAbs by IBT and which could not be detected on TYCSB were isolated from the replicate blood agar plate for further identification. Between two and five colonies which had colony morphology on blood agar similar to that of mutans streptococci but which did not react with the IBT were also isolated from blood agar plates for further identification. All colonies were identified with MAbs and by comparing their cell wall protein patterns with those of reference strains by SDS-PAGE (26, 27).

To study the presence of *Streptococcus rattus* and *S. cricetus* in plaque samples, strains which were positive for OMVU2 and negative for other MAbs were isolated. *S. rattus* strains were those which possessed arginine dehydrogenase activity (API 20 Strep; Analytab Products, Montalieu-Vercieu, France). *S. cricetus* strains were those OMVU2-positive strains which reacted with OMVU40.

RESULTS

IBT. The IBT was first tested with nitrocellulose membranes which were placed on the blood agar plates after the plates had been incubated in order to transfer portions of bacterial colonies. These membranes showed sometimes vague dots after the immunostaining was performed. To obtain distinct dots after the staining procedure, the bacteria were grown on the membranes. An example of a nitrocellulose membrane inoculated with a plaque sample and incubated as described is shown in Fig. 1. The IBT was tested with laboratory strains, in which the recoveries of four *S. mutans*, four *S. sobrinus*, two *S. cricetus*, and two *S. rattus* strains by the IBT and by culturing on blood agar plates were virtually identical. The recovery by the IBT compared to that on blood agar was 103% ± 15% (mean ± standard deviation) for 16 independent experiments (data not shown).

Several oral bacteria reacted with the horseradish peroxidase-conjugated second antibodies. Antibodies from 10 different manufacturers were tested, and the particular second antibodies described here showed some cross-reactions with *Capnocytophaga* species, *Bacteroides gingivalis*, and some yeasts. To prevent counting these bacteria as mutans streptococci, second antibody cross-reactivity was always measured prior to MAb incubation. Second antibody-positive colonies were detected in 75% of the plaque samples. The numbers of cross-reactive colonies varied widely, ranging between 1 and 40% (mean, 3%) of the total viable counts on blood agar.

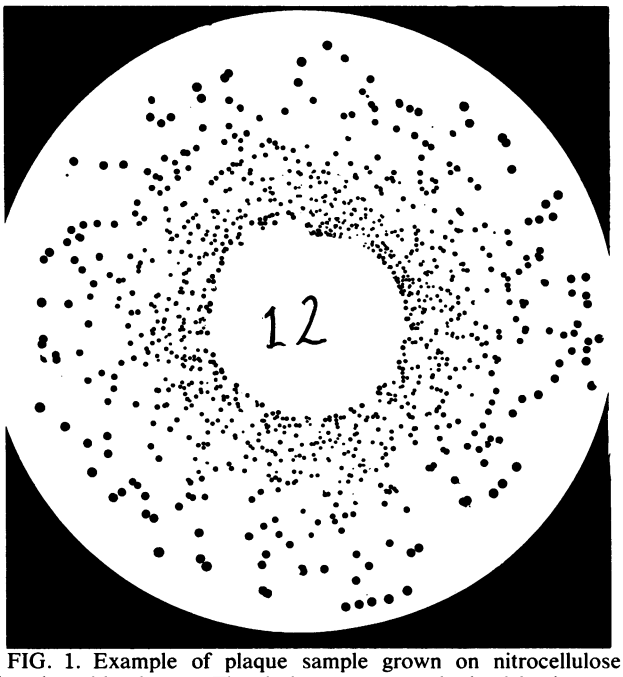

FIG. 1. Example of plaque sample grown on nitrocellulose, placed on blood agar. The dark spots were obtained by immune staining with OMVU10, OMVU31, and OMVU2 and with a horseradish peroxidase-conjugated second antibody, as described in the text.

Rat dental plaque. The results of the bacterial counts of the dental plaque samples from the rats are shown in Fig. 2. The percentages of *S. mutans* in the plaque samples on TYCSB ranged from 25 to 68% of the total numbers of CFU. With the IBT, the numbers ranged from 30 to 90% of the total numbers of CFU on blood agar. Statistical analysis showed that significantly higher numbers of *S. mutans* were detected with the IBT than on TYCSB ($P = 0.016$, paired Student's t test). The mean recovery of these *S. mutans* strains on TYCSB compared with that by the IBT was 82% ± 10%.

S. sobrinus numbers ranged from 1 to 60% of total numbers of CFU on TYCSB and from 25 to 90% of total numbers of CFU with IBT. Significantly higher numbers of *S. sobrinus* were counted with IBT than on TYCSB ($P = 0.007$, paired Student's t test). The mean recovery of the *S. sobrinus* strains on TYCSB was 37% ± 30% of the counts with the IBT.

Human dental plaque samples. The results of the bacterial counts on TYCSB and with the IBT are shown in Fig. 3. In 40 plaque samples, *S. mutans* was detected in higher numbers with the IBT than on TYCSB. In 15 of these samples, we found over 10 times as much *S. mutans* with the IBT. In eight samples, culturing on TYCSB resulted in more *S. mutans* colonies than with the IBT. However, some of these plaque samples (Fig. 3, samples 4 through 8) harbored large numbers of *S. sobrinus* with the IBT. The morphology of these *S. sobrinus* colonies on TYCSB was similar to the morphology of *S. mutans*, and therefore, these colonies were incorrectly identified as *S. mutans*. In three samples, *S. mutans* was not detected on TYCSB, while with the IBT, more than 10^4 CFU per sample were found. In one sample (sample 5), no *S. mutans* was detected with the IBT, while more than 10^5 CFU were found on TYCSB. In three samples, we were not able to detect *S. mutans* by either method. These results indicate that significantly higher numbers of *S. mutans* were counted with the IBT than with TYCSB ($P < 0.001$, paired two-tailed Student's t test).

In 26 plaque samples, no *S. sobrinus* colonies were detected on TYCSB, but all were positive with the IBT. No plaque samples were encountered which were positive for *S. sobrinus* on TYCSB but negative with the IBT. In seven samples, *S. sobrinus* was not detected by either method, and in one sample, growth on TYCSB revealed slightly more colonies than with the IBT. So, *S. sobrinus* was detected more frequently with the IBT than on TYCSB ($P < 0.005$, chi-square test), and most plaque samples had higher *S. sobrinus* counts by using IBT ($P < 0.001$, paired two-tailed Student's t test). This was found for plaque samples with high numbers of *S. sobrinus* as well as for samples with low numbers of *S. sobrinus*.

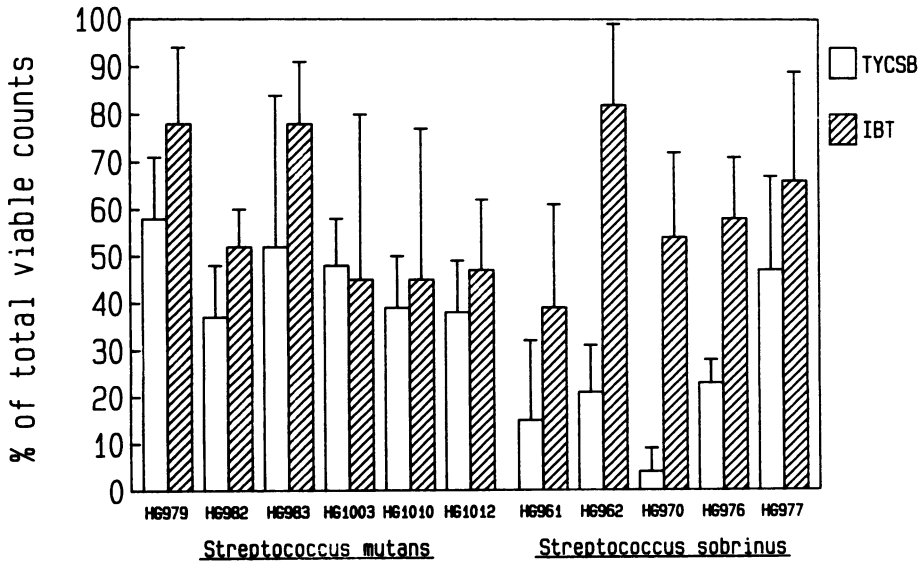

FIG. 2. Recovery of *S. mutans* and *S. sobrinus* strains from rat molar homogenates on TYCSB and by IBT. The data are presented as percentages of the total anaerobic viable counts on blood agar.

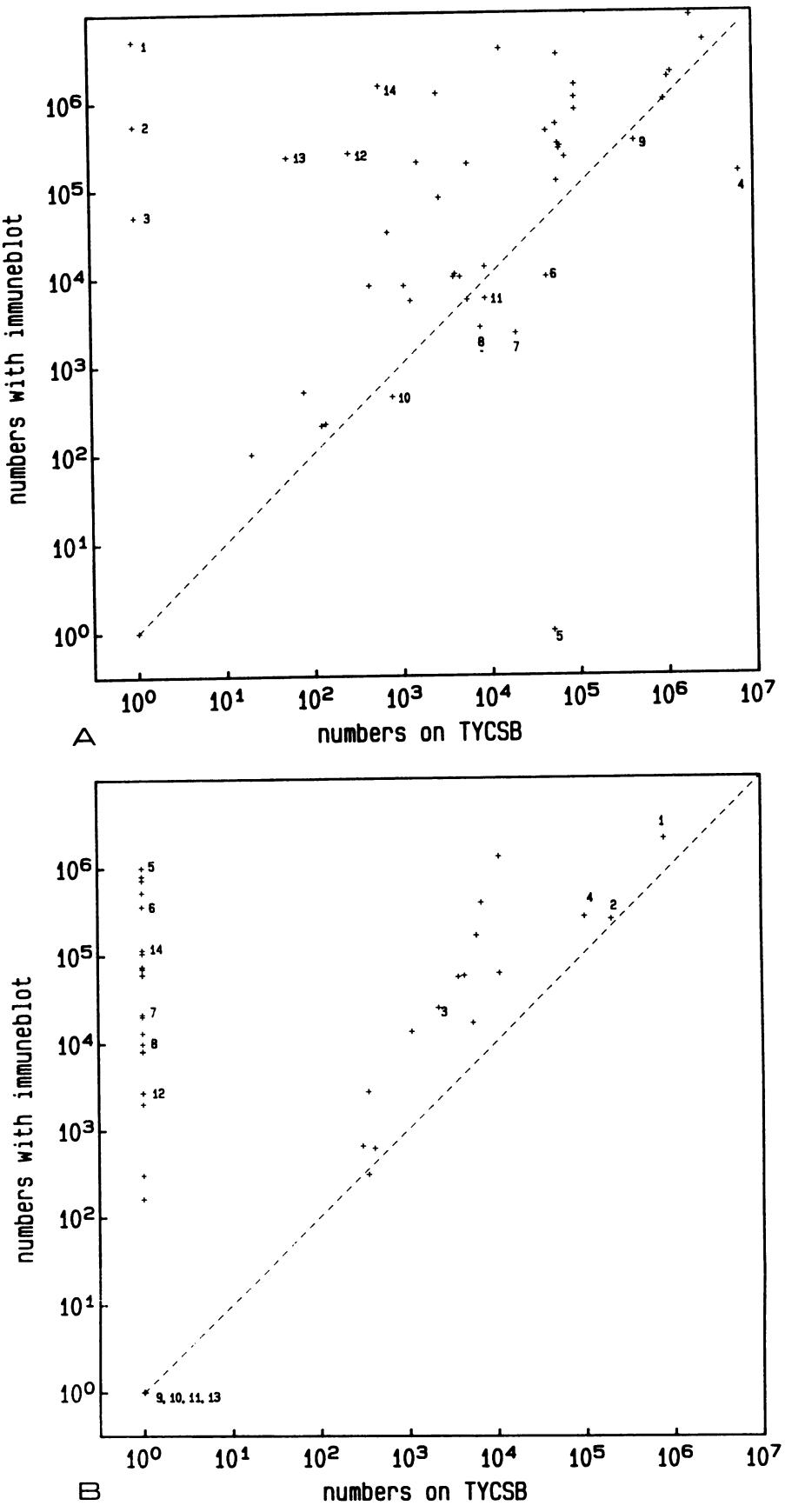

FIG. 3. (A) Scattergram of the numbers of *S. mutans* in human dental plaque, obtained by culturing on TYCSB (x axis) and by IBT with OMVU31 and OMVU2 (y axis). (B) Scattergram of the numbers of *S. sobrinus* in human dental plaque, obtained by culturing on TYCSB and IBT with OMVU10. The digits in both figures are the numbers of corresponding plaque samples, in order to allow comparison of *S. mutans* counts and *S. sobrinus* counts in the same plaque sample.

To check whether false-positive or false-negative reactions could possibly have occurred with the IBT, several colonies of each sample were isolated and identified. Two to five colonies that had reacted with one of the MAbs by the IBT per sample were isolated from blood agar plates and identified. This was also performed with colonies which did not react with one of the MAbs but which had a colony morphology resembling that of mutans streptococci. The reactivity of these strains with MAbs after they were isolated from blood agar was identical to the reactivity with the IBT, meaning that both *S. mutans* and *S. sobrinus* reacted with MAbs and other bacteria did not. This was in agreement with the cell wall protein patterns determined by SDS-PAGE, with type strains of *S. mutans* and *S. sobrinus* as controls. Colonies which reacted only with OMVU2 did not possess arginine dehydrogenase activity and were not reactive with OMVU40. Cell wall protein patterns determined by SDS-PAGE that were similar to reference strains of *S. rattus* or *S. cricetus* were not detected.

Second antibody cross-reactivity was observed in 75% of the samples. Therefore, colonies which reacted with the second antibodies were detected prior to MAb incubation.

DISCUSSION

Coykendall described the genetic heterogeneity within the group of mutans streptococci and proposed new species (6–8). To determine the role of the different mutans streptococcal species in dental caries, these species should be studied separately. This has been performed previously with polyclonal antisera (1–4, 11, 14, 24, 25). However, good antisera, which are not cross-reactive with other bacteria and have high titers, are difficult to obtain. Therefore, most epidemiological studies describe the presence of mutans streptococci on the basis of growth and colony morphology on selective media. These selective media, however, can inhibit the growth of *S. sobrinus* (18, 30, 31, 35). Many epidemiological studies may be hampered by this problem.

The identification of *S. mutans* and *S. sobrinus* is not possible with colony morphology alone. Identification by sugar fermentation reactions is impeded, however, by differences in sugar fermentation patterns within one species. Correct identification of oral mutans streptococci is possible by using DNA studies (G+C content determinations and homology studies). However, these techniques are laborious and not easy to perform. SDS-PAGE cell wall pattern analysis gives a reliable identification also, but it is generally limited to the capacity of gel systems available. There are also identification kits commercially available. Although these kits give easy identification of oral streptococci, the results are not reliable (10). To create a correct and easy identification system, MAbs have been used (10a). Immunofluorescence techniques are commonly used to detect mutans streptococci, but until now we have not been able to use MAbs for this purpose. The OMVU10 MAbs did not react with *S. sobrinus* directly in dental plaque samples in a protocol, as published previously (10). Therefore, the bacterial samples were cultured prior to immunological detection and identification. It has been reported that the recovery of *S. sobrinus* is better on TYCSB than on other selective media, though not as good as culturing on nonselective media (28, 35). Controversially, Svanberg and Krasse (29) reported that the recovery of *S. sobrinus* on MSB was better than the recovery on TYCSB. This phenomenon might be due to one of the bases used to prepare this medium. The particular proteose peptone (Difco) which was used by van Palenstein Helderman et al. (34) has not always been used by others. This greatly decreases the recovery of *S. sobrinus* on TYCSB (W. P. Holbrook, personal communication). Therefore, a nonselective culturing method was combined with an IBT.

The IBT used on plaque samples from rats showed that *S. sobrinus* is extremely underestimated (37% ± 26%) and that *S. mutans* was marginally underestimated (82% ± 12%) on TYCSB, compared with the IBT.

In order to test the IBT for use in epidemiological studies, 51 human dental plaque samples were investigated. All samples detected on TYCSB which contained *S. sobrinus* or *S. mutans* were also positive with the IBT. This means that no positive *S. sobrinus* or *S. mutans* samples were missed. Only one sample (Fig. 3A, sample 5) had higher counts on TYCSB than by the IBT. This sample contained an *S. sobrinus* strain which was morphologically identical to *S. mutans* on TYCSB and was therefore mistaken for *S. mutans*. In 26 samples, *S. sobrinus* was detected with the IBT in combination with OMVU10, while *S. sobrinus* could not be found on TYCSB. This phenomenon was not due to false-positive reactions, because SDS-PAGE patterns of the cell wall proteins of these colonies were identical to those of a reference *S. sobrinus* strain. Some of the differences in numbers of *S. sobrinus* between TYCSB and the IBT can be explained by misidentification on TYCSB using colony morphology. From four of these plaque samples (samples 5, 6, 7, and 8), we were able to isolate *S. sobrinus* from blood agar as well as from TYCSB by picking several colonies with a slightly different colony morphology. These strains had a colony morphology similar to that of *S. mutans* and therefore were not identified as *S. sobrinus*. We concluded that most of these *S. sobrinus* strains did not grow initially on TYCSB. After subculturing on blood agar plates, these strains were able to grow on TYCSB plates. This observation is supported by the recovery of strain HG962 from rat plaque samples, as this strain is almost completely inhibited on TYCSB (Fig. 2).

Differences between both methods were also found with respect to the recovery of *S. mutans*. Of 51 samples, 15 gave counts of *S. mutans* with the IBT that were more than 10 times higher than those on TYCSB. On the basis of SDS-PAGE, we concluded that this increase in numbers of *S. mutans* was not due to false-positive reactions. With the IBT, we were able to count higher numbers of *S. mutans*, but positive plaque samples remained positive and negative plaque samples remained negative. A few plaque samples contained large numbers of *S. sobrinus*. The results suggest that in those cases the high numbers of *S. sobrinus* on TYCSB probably interfered with proper counting of *S. mutans*. Other *S. mutans* strains were strongly inhibited on TYCSB. The IBT overcomes these problems. Growth on nitrocellulose membranes might limit the IBT. However, we were not able to detect inhibition of growth by using laboratory strains. The numbers of colonies on blood agar and nitrocellulose were identical (data not shown).

The IBT cannot detect *S. cricetus* and *S. rattus* directly. *S. cricetus* cannot be detected because the MAbs specific for this species (OMVU40) are cross-reactive with *Streptococcus gordonii*. This bacterium is often found in human dental plaque. Thus, *S. cricetus* had to be detected on the basis of reactivity with two different MAbs, OMVU2 and OMVU40. *S. cricetus* was not found in the 51 plaque samples of this study. All strains which reacted with OMVU2 showed negative reactions with OMVU40. Also, no OMVU2-positive strains were found after isolation and subculturing of

OMVU40-positive strains. *S. rattus* cannot be detected because we do not have specific MAbs against *S. rattus*. However, no arginine dehydrogenase-producing mutans streptococci were found in the 51 plaque samples of this study.

In conclusion, the enumeration of mutans streptococci from dental plaque or saliva is possible with the IBT by using the MAbs OMVU2, OMVU10, and OMVU31. We did not detect false-positive or false-negative reactions. This method gives a more reliable picture of the number and identity of *S. mutans* and *S. sobrinus* in clinical samples than using selective media like TYCSB does. With this method, epidemiological studies on the prevalence of these bacteria are in progress.

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