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IMPLANTATION OF *STREPTOCOCCUS MUTANS* ON TOOTH SURFACES IN MAN

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Summary—Streptomycin-resistant strains of *Streptococcus mutans* were implanted as adherent growth on artificial fissures (AF). A stock culture of strain E49 (serotype a) and human isolates of serotype c strains were each implanted in three subjects. The AF was retained in the occlusal surface of a tooth for 80–288 h, during which occlusal samples from the AF and salivary samples were regularly cultured, as well as AF itself when removed from the mouth. The streptomycin-resistant marker appeared stable *in vitro* and *in vivo*. Strain E49 failed to establish, as the colony-forming units (CFU) of this organism decreased with time in the AF to low or undetectable levels. At the same time, the AF became colonized by the indigenous flora including streptomycin-sensitive strains of *Strep. mutans*. The implanted serotype c strains of *Strep. mutans* seemed to establish; the CFU of these strains in the AF did not appreciably decrease with time *in vivo*. This suggests that serotype c strains possess a unique advantage over a stock culture of a serotype a strain in their ability to implant in the human mouth using the AF model. Although the implanted *Strep. mutans* strains were always found in the saliva when their numbers exceeded 10^5 CFU in the AF, they were rare when the level was below 10^5 CFU. In samples from the AF orifice, implanted *Strep. mutans* was only detected on 5 of 18 occasions, when these strains were demonstrably present within the AF.

INTRODUCTION

Streptococcus mutans is a dental pathogen in animal models (Keyes, 1968) and is statistically associated with human dental decay (Krasse *et al.*, 1968; Loesche *et al.*, 1975); it appears to colonize the oral cavity when teeth erupt (Carlsson *et al.*, 1970; Berkowitz, Jordan and White, 1975). Most pre-school children have detectable levels of *Strep. mutans* in their dental plaque (Catalanotto, Shklair and Keene, 1975) and the mouths of older children are almost universally colonized by this organism (Loesch *et al.*, 1975). This is in contrast with adults, where the establishment of *in-vitro* grown strains carrying a streptomycin-resistant marker is difficult to accomplish (Krasse *et al.*, 1967; Jordan *et al.*, 1972; Edman *et al.*, 1975). Krasse *et al.* (1967) used various methods to enhance colonization, i.e. the tooth surfaces were mechanically polished, the subjects were given multiple inoculations as mouthrinses and sucrose was ingested frequently. Edman *et al.* (1975) used dental floss impregnated with *Strep. mutans*. The labelled strains gradually disappeared from the oral cavity.

Strep. mutans seems to establish preferentially in fissures (Ikeda and Sandham, 1971; Gibbons *et al.*, 1974; Berkowitz, Jordan and White, 1975). Our aim was to implant streptomycin-resistant *Strep. mutans* in fissure sites using both the natural tooth and an artificial model.

MATERIALS AND METHODS

Strep. mutans *strains*

Stock cultures of E49 (serotype a), FA1 (serotype b), GS5 (serotype c) and LM7 (serotype e), as well as isolates of *Strep. mutans* strains G, T and S (serotype c) obtained from the dental plaque of volunteers, were serially passed on MM10 sucrose agar plates (Syed and Loesche, 1973) containing increasing levels of streptomycin, until mutants capable of growth in the presence of 1 mg/ml of streptomycin were obtained. Strain 6715 (serotype d) was already resistant to streptomycin.

Implantation

Implantation on a natural surface. Eight adults volunteered to have streptomycin-resistant strains placed on a tooth surface. Stock cultures of the serotypes were grown overnight in a 1 per cent trypticase, 0.2 per cent yeast extract broth containing either 0.5 per cent glucose or 0.5 per cent sucrose. The cultures were centrifuged and the pellet re-suspended in 1 ml of reduced transport fluid (RTF) (Syed and Loesche, 1972) containing 1 per cent sucrose. A tuft of sterile cotton was immersed in the suspension and placed on the occlusal surface of a wiped-dry molar tooth. The tuft containing the inoculum was retained on the occlusal surface for 3 h by a sheet of soft occlusal wax placed over the occlusal surface and adapted to the teeth. Salivary and occlusal samples were cultured immediately after the removal of the wax and cotton tuft and again every morning for the following 7 days.

Implantation in an artificial fissure. All of the six adult subjects (subjects C, W, M, G, T and S) who participated needed a gold crown on a first molar tooth. The crown was prepared with a recess on its occlusal surface which could accept a rectangular insert. A Mylar bag resembling the natural occlusal fissure was supported in a cast gold inlay, or insert, fabricated as described by Loe, Karring and Theilade (1973) (Fig. 1). The insert containing the Mylar bag will be termed here "artificial fissure (AF)".

Overnight cultures of the stock streptomycin-resistant (Sm^R) *Strep. mutans* strains were centrifuged and resuspended in 1 ml of RTF. Five microlitres of this

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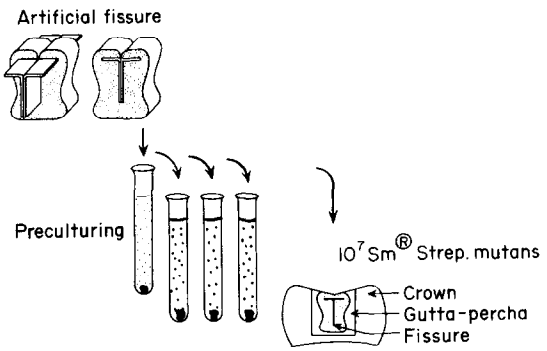

Fig. 1. Culturing scheme used to obtain adherent growth of streptomycin-resistant (Sm^R) strains of *Strep. mutans* on artificial fissures.

suspension was injected into a sterile AF *in vitro*, whereafter the AF was immediately placed in the tooth and secured with gutta-percha. In most experiments, the Sm^R strain of *Strep. mutans* and the AF were cultured overnight in 10 ml of trypticase-soy broth (TSB) containing 0.1 per cent sucrose for 3 consecutive days. The AF with adherent growth of *Strep. mutans* strains was placed in the recess on the gold crown and retained with gutta-percha (Fig. 1).

Implantation with serotype a strains of Strep. mutans *in the AF.* An AF containing a suspension of E49 was inserted for 48–168 h on five occasions in subject W, that with adherent growth of E49 in subject C for 80, 90, 160 and 220 h, in subject W for 80, 160 and 220 h and in subject M for 80, 220 and 240 h.

Implantation wih serotype c strains of Strep. mutans *in the AF.* Strains G, T and S, which were originally isolated from subjects G, T and S respectively, were implanted back into the donor's mouth as adherent growth on the AF, for 168, 216 and 288 h. Strain S was also implanted in subject W on 5 occasions, in subject G on 3 occasions and in subject T on 2 occasions for periods varying from 90 to 288 h.

Bacterial procedure

Unstimulated saliva was collected before the insertion of the AF and at various times thereafter. Occlusal samples were obtained *in vivo* from the orifice of the AF with a 26-gauge needle held in a haemostat. The AF was removed from the mouth at certain time-intervals. The Mylar bag was separated from the gold inlay and the contents cultured. The samples were immediately placed in RTF and thereafter dispersed by sonification (Branson Model W 185 D, New York) for 5 s. They were then serially diluted in RTF and 0.05-μl aliquots from appropriate dilutions were placed, in duplicate, on mitis-salivarius bacitracin agar (MSB) (Gold, Jordan and van Houte, 1973) and on MM10 sucrose agar (Syed and Loesche, 1973) with and without 0.2 mg/ml of streptomycin. All plates were inoculated within 30 min of collection of samples and were immediately placed in the anaerobic chamber and incubated at 37°C for 48 h in an atmosphere of 85 per cent N_2, 10 per cent H_2 and 5 per cent CO_2.

Colonies of *Strep. mutans* strains were identified on the various media by their characteristic colony morphology, supplemented if necessary with biochemical tests (Shklair and Keene, 1974) and by examination with fluorescent antibody directed against the various *Strep. mutans* serotypes (Grenier, Eveland and Loesche, 1973). Total colony-forming units (CFU) counts and *Strep. mutans* counts were obtained from the MM10 sucrose agar. The *Strep. mutans* counts were also obtained from the MSB agar. The counts of Sm^R strains were obtained from the MM10 sucrose streptomycin agar. All counts were converted to $\log_{10}$ for depiction in figures.

RESULTS

Implantation of various serotypes of Strep. mutans *strains on a natural tooth surface*

All attempts to establish the various serotypes of Sm^R *Strep. mutans* strains on a natural tooth surface failed. The implanted strains were not detected in the saliva or on the tooth surface in any subject after one day.

Implantation of serotype a Strep. mutans *in an AF*

Strain E49 was introduced into the mouth via the AF in two ways. Initially, approximately 10^5 CFU were syringed into the AF *in vitro*, then the AF was placed *in vivo*. No CFU of E49 could be detected in the AF when it was removed from the mouth after 72–120 h. At removal, the AF was colonized by 10^6–10^7 microorganisms including streptomycin sensitive (Sm^s) strains of *Strep. mutans*.

In the second approach, the AF was serially passed in sucrose broth with E49 so as to obtain adherent growth of the *Strep. mutans* strain on the AF. The AF, at the time of insertion into the mouth of the 3 subjects, contained an average of 10^7 CFU of strain E49 (range: 1.26×10^6–2.51×10^7 CFU, $n = 20$). These AF levels decreased over a 240-h period to undetectable or low levels in subjects C and M and to about 10^5 CFU in subject W (Fig. 2).

On all occasions in subjects C and W, and on one of three occasions in subject M, the AF became colonized by Sm^S strains of *Strep. mutans* (Fig. 2). The numbers of these presumably endogenous organisms increased in the AF with time. On the two occasions in subject M, that the Sm^S strains failed to appear in the AF (Fig. 2), the average salivary concentrations were below 10^3 CFU per ml. Subject M during this period of low salivary *Strep. mutans* levels had a low sucrose intake and was not eating between meals. Subsequently, salivary *Strep. mutans* levels in subject M increased to about 1.4×10^4 CFUs per ml (Fig. 2) when frequent food snacks were taken. The single AF placed into his mouth during this time was readily colonized by his endogenous *Strep. mutans*. The average salivary concentrations of endogenous *Strep. mutans* in subjects C and W when the AFs were being colonized by Sm^S organisms was 3.1×10^4 CFU per ml in subject C and 5.0×10^5 in subject W (Table 1).

The implanted strain E49 was shed from the AFs into the saliva (Fig. 2). The salivary levels of strain E49 in subjects C and M decreased with time from about 10^5 CFUs immediately after insertion of the AF to undetectable levels, i.e. less than 10^1 CFUs per ml, within 85–160 h (Figs. 2a and 2c). In subject W, the salivary concentration of strain E49 also de-

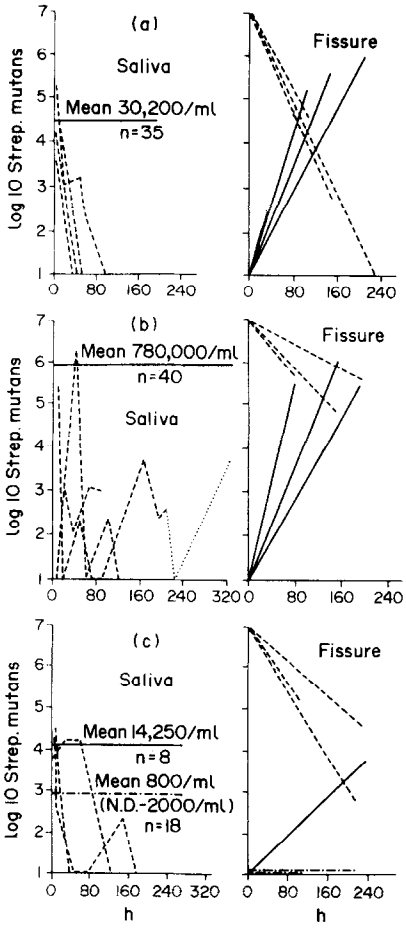

Fig. 2. Concentration (log 10) of streptomycin-resistant strain E49 and streptomycin-sensitive strains of *Strep. mutans* in the salivas and in the artificial fissures of three subjects: subject C(a); subject W(b); subject M(c). Symbols used: solid line represents values for streptomycin-sensitive *Strep. mutans* in the saliva and AF; broken line represents values for streptomycin-resistant strain E49 in saliva and AF; dotted line represents salivary concentrations of strain E49 after removal of the AF; dash–dot–dash line represents streptomycin-sensitive *Strep. mutans* in the saliva of subject M(c) during a period when the AF did not become colonized by this organism.

creased, but showed an oscillating pattern between 10^1 to 10^4 CFUs per ml and, on one occasion, was detected 114 h after the removal of the AF (Fig. 2b).

The disappearance of strain E49 from the AF and saliva was coincident with the increase of Sm^S strains of *Strep. mutans* in the AF (Fig. 2), suggesting that strain E49 may have back-mutated and lost its Sm^R marker *in vivo*. This possibility was ruled out by examination of the number of CFUs of *Strep. mutans* which grew on the MSB, MM10 sucrose agar with streptomycin and MM10 sucrose agar without streptomycin. Strain E49, like all serotype a strains of *Strep. mutans*, is bacitracin sensitive and therefore does not grow on MSB agar (Shklair and Keene, 1974). Endogenous strains of *Strep. mutans* are primarily of serotypes c, d and e (Bratthall, 1972; Thomson, Little and Hageage, 1976; Loesche and Grenier, 1976) and will grow on MSB agar (Shklair and Keene, 1974). E49 can therefore be distinguished from any E49 Sm^S mutants and from endogenous strains of *Strep. mutans* (Table 2). The Sm^S *Strep. mutans* strains that displaced strain E49 in the AF grew on MSB agar and were therefore presumably of endogenous origin, i.e. the streptomycin-resistant marker appeared to be stable *in vitro* and *in vivo*.

Implantation of serotype c strains of Strep. mutans *in an AF*

Subjects G, T and S were inoculated with an AF containing adherent growth of Sm^R strains G, T and S of *Strep. mutans*, originally isolated from the subject's own mouth. All AFs had detectable levels of Sm^R strains at their removal from the mouth (Fig. 3). In subject G on one occasion (Fig. 3a), in subject T on 2 occasions (Fig. 3b) and in subject S on 3 occasions (Fig. 3c), the final levels of the Sm^R strains of *Strep. mutans* in the AF were essentially the same as the starting levels.

In subjects G and T, all AFs were at the time of removal also colonized by Sm^S strains of *Strep. mutans* (Figs. 3a and b). In subject S, on no occasion could any Sm^S *Strep. mutans* strains be detected (Fig. 3c). The average salivary concentrations of these endogenous Sm^S strains of *Strep. mutans* were approximately 3.7×10^3 CFUs per ml in subject G, 1.6×10^4 in subject T and 1.4×10^6 in subject S (Table 1, Fig. 3).

The implanted strains G, T and S shed from the AFs into the saliva. Their salivary concentrations varied from about 10^5 CFUs per ml immediately after implantation to about 10^1–10^3 CFUs at the time of removal of the AF from the mouth. In one experiment in each subject, the saliva was repeatedly cultured for the Sm^R strains after the AF was withdrawn from

Table 1. Salivary concentrations of streptomycin-sensitive *Strep. mutans*

Subject	*Strep. mutans* CFU/ml average	Range	No. of samples
C	3.16×10^4	2.0×10^3–1.6×10^5	35
W	7.8×10^5	3.1×10^4–1.0×10^7	40
	8.0×10^2	$<10^2$–2.0×10^3	18
M			
	1.4×10^4	10^2–3.2×10^4	8
G	3.7×10^3	$<10^2$–2.0×10^4	45
T	1.6×10^4	7.9×10^3–4.0×10^4	31
S	1.4×10^6	6.3×10^4–6.3×10^7	50

Table 2. Antibiotic-resistance scheme to determine whether streptomycin resistant serotype a strains of *Strep. mutans* lose this marker *in vivo*

Growth on	MSB agar	MM10 sucrose agar	
		With streptomycin	Without streptomycin
E49 Sm^R	−	+	+
E49 Sm^S	−	−	+
Endogenous *Strep. mutans* Strains	+	−	+
G, T and S	+	+	+

the mouth. In each instance, the salivary levels decreased to barely detectable values or were undetectable (Fig. 3).

The AF-containing adherent growth of strain S did not become colonized by endogenous *Strep. mutans*, even though subject S had the highest salivary *Strep. mutans* level (Table 1). This absence of colonization could reflect some inhibitory attribute of strain S or could be due to the inability in subject S of endogenous *Strep. mutans* to colonize the AF. In additional experiments, subjects G, T and W were inoculated with strain S as adherent growth in the AF. When the AFs were removed after 90–288 h *in vivo*, they contained about 10^4–10^7 CFUs of strain S. As in subject S, no Sm^S *Strep. mutans* could be detected among the indigenous microorganisms that had colonized the AF.

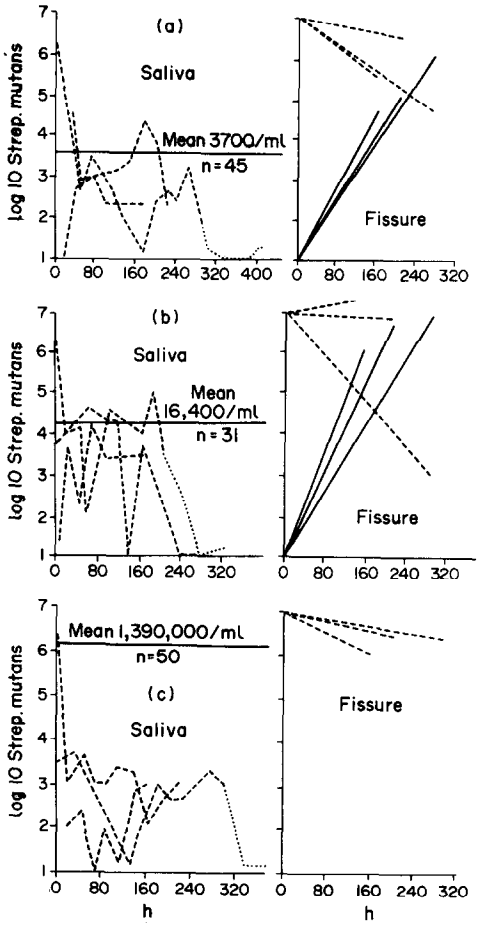

Fig. 3. Concentrations of streptomycin-resistant strains, i.e. strain G in subject G(a), strain T in subject T(b) and strain S in subject S(c), in the saliva and in the AF of three subjects. Symbols used: solid line represents values for streptomycin-sensitive *Strep. mutans* in saliva and AF; dashed line represents values for Sm^R *Strep. mutans* in saliva and AF; dotted line represents salivary concentrations of Sm^R *Strep. mutans* after removal of the AF.

Relationship between the salivary and AF concentrations of the implanted serotype c strains of Strep. mutans

Saliva samples were always collected from subjects G, T, and S immediately before the removal and culturing of the AF from the tooth. When the data from the 3 subjects were combined, a relationship between the levels of Sm^R strains in the AF and in the saliva was detected. When the total number of the Sm^R *Strep. mutans* strains was below 10^5 CFUs in the AF (Fig. 4), these strains were detected in only 1 of 9 salivary samples. However, when the number of Sm^R *Strep. mutans* in the AF exceeded 10^5 CFUs, they were always found in the salivary samples, but its numbers per ml were 2.5–4 log lower than its total number in the AF.

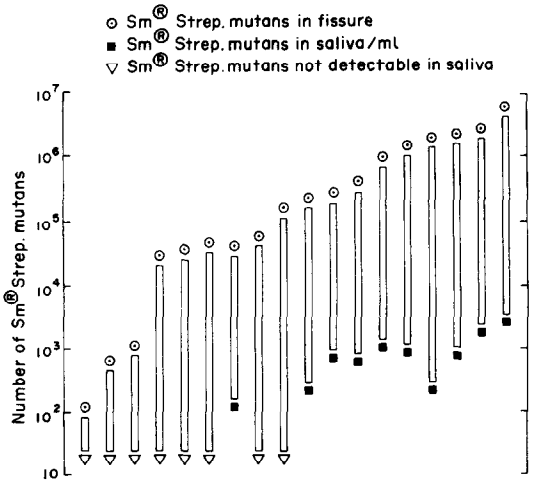

Fig. 4. The relationship between AF levels of streptomycin-resistant (Sm^R) *Strep. mutans* and the salivary levels of these organisms. Note that when the fissure levels were $\geq 10^5$ CFUs the Sm^R strains were always detectable in the saliva.

Relationship between samples positive for the implanted serotype c strains of Strep. mutans *from the orifice of the AF and from the total AF*

The orifice of the AF was sampled immediately before the removal of the AF. Streptomycin-resistant *Strep. mutans* strains were detected on only 5 out of 18 occasions when these strains were demonstrably present in the AF.

DISCUSSION

Our findings confirm and extend previous investigations concerning the difficulty of implanting *in-vitro* grown, streptomycin-resistant strains of *Strep. mutans* in the mouths of adults (Krasse *et al.*, 1967; Jordan *et al.*, 1972). All attempts to establish the various serotypes by implantation on a natural tooth surface failed. Failure to detect the Sm^R strain could mean that either it was rapidly cleared from the mouth, or that it had back-mutated and lost its streptomycin-resistant marker. The Sm^S strains of *Strep. mutans* which colonized the AFs were all resistant to bacitracin, indicating that they were not mutants of strain E49, unless an extremely rare double mutation had occurred. It was more likely, therefore, that the endogenous strains of *Strep. mutans* were able to compete extremely well in the AF, whereas strain E49, given an initial advantage, could not.

Strain E49 persisted in only one subject (Fig. 2b). Serotype c human isolates of *Strep. mutans* isolated from the subjects' own mouths, however, was established in all cases (Fig. 3). In 6 out of 9 experiments, the total CFU of the implanted strains G, T and S in the AF at the time of removal from the mouth was approximately the same as when the AF was introduced. Strain S was successfully implanted in 4 individuals. This would suggest that serotype c strains possess a unique advantage over serotype a strain E49 in their ability to implant in the human mouth using the AF model. This agrees with the relative absence of serotype a strains of *Strep. mutans* and the predominance of serotype c strains in man (Bratthall, 1972; Thomson *et al.*, 1976; Loesche and Grenier, 1976). Strain E49 was originally isolated from hamster teeth (Fitzgerald and Keyes, 1960). Krasse *et al.* (1967) found a higher clearance from the human mouth of the serotype a, hamster *Strep. mutans* strain 3720 than that of the human serotype c *Strep. mutans* strain Ingbritt. This suggests that man possesses salivary factors directed against serotype a and/or that serotype c strains possess some attribute(s), possibly a surface receptor, that gives them an ecological advantage over serotype a strains when both are introduced into the mouth. Strain E49 has been maintained by laboratory transfer for 15 years, possibly resulting in changes which could be of some significance for the ecological differences observed between the serotype a and the recently-isolated serotype c strains (Gibbons and Fitzgerald, 1969; Ellwood *et al.*, 1976). These changes could be tested for by studies using recent human isolates of serotype a and old stock cultures of a serotype c strain. Also, within the serotype c, *Strep. mutans* strains differences may exist that influence establishment. When strains G and T were introduced as *in-vitro* cultured adherent growth in the AF, streptomycin-sensitive strains of *Strep. mutans* readily colonized the AF in numbers increasing with time. When strain S was implanted using the same technique in 4 separate individuals, no Sm^S *Strep. mutans* colonized the AF, indicating that some characteristic of strain S inhibited the colonization.

Svanberg and Loesche (1977) showed that the AFs become colonized by endogenous *Strep. mutans* at salivary levels of $\geq 10^3$ CFUs per ml. We found that, with the exception of the AFs containing strain S, all AFs became colonized by endogenous *Strep. mutans* whenever this organism was $\geq 10^3$ CFUs per ml of saliva. In subject M, when his saliva contained a mean of 800 CFUs of *Strep. mutans* per ml, the AFs did not become colonized with this organism.

The bacteriological results of samples from the saliva and AF orifice, which were collected immediately before the removal of the AF, show the difficulty in detecting absolutely a *Strep. mutans* infection in a retention site such as a fissure. When more than 10^5 Sm^R CFU of *Strep. mutans* were present in the AF, they were always detectable in the saliva. When the AF contained less than 10^5 Sm^R CFU of *Strep. mutans*, only 1 out of 9 salivary samples was positive for these microorganisms. In samples taken from the orifice of the AF, the Sm^R strains of *Strep. mutans* were undetected in 13 out of 18 occasions in which these strains were demonstrably present in the contents of the AF. These findings illustrate one reason why tests based upon detection of *Strep. mutans* strains have only a relative predictive value as an indicator of preclinical caries (Svenson, Liljemark and Schuman, 1976).

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