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Effect of Chlorhexidine Varnish on Actinomyces naeslundii Genospecies in Plaque from Dental Fissures

Abstract
This study describes the effects of varnish containing 40% chlorhexidine diacetate on Actinomyces naeslundii populations in plaque from human molar fissures. In each of 15 subjects two dental fissures with high levels of mutans streptococci were selected. The experimental treatment consisted of the single application of a small amount of chlorhexidine varnish onto the selected fissures. The varnish was removed 15 min after application. One month after varnish application a significant increase was observed in A. naeslundii counts while the number of mutans streptococci had decreased significantly compared with preexperimental levels. From 85 randomly selected Actinomyces isolates taken from blood agar plates before varnish application, 44% belonged to A. naeslundii genospecies 1 and 56% to A. naeslundii genospecies 2. From 106 isolates taken 1 month after chlorhexidine varnish application, 42% belonged to A. naeslundii genospecies 1 and 58% to A. naeslundii genospecies 2. At baseline 28% of A. naeslundii genospecies 1 strains were catalase-positive, but 1 month after varnish application 4% of the strains were catalase-positive (p <0.05). It is concluded that chlorhexidine varnish application caused an increase of A. naeslundii in dental plaque, but induced no significant changes in the distribution of the two A. naeslundii genospecies.

Several studies have shown that treatment of tooth surfaces with chlorhexidine varnish can lead to a long-lasting suppression of mutans streptococci, and subsequent caries reduction [reviewed by Emilson, 1994]. Microbiological monitoring following chlorhexidine varnish treatment suggested that this long-term suppression of mutans streptococci is mainly due to bacterial interference [Schaeken et al., 1989]. Earlier studies indicated that streptococci from the Streptococcus oralis group, such as Streptococcus gordonii, S. oralis, Streptococcus sanguis and Streptococcus mitis, and Actinomyces species play a role in the suppression of mutans streptococci [Mikx et al., 1975; van der Hoeven and Rogers, 1979; McDermid et al., 1987; Perrons and Donoghue, 1990]. Indeed, using gnotobiotic rats it could be demonstrated that Streptococcus and Actinomyces strains inhibit regrowth of Streptococcus mutans after chlorhexidine application [van der Hoeven and Schaeken, 1995]. It is not surprising that physiologically related bacteria are involved in control of mutans streptococci in dental plaque. However, it is not known how specific the relationship is, and if particular Streptococcus and Actinomyces species have more effect than others in controlling mutans strepto-
coci. In an earlier experiment we have recorded the *Streptococcus* species that repopulate the tooth surface after chlorhexidine varnish application [Schaeken et al., 1994]. It appeared that the sequence of recolonization of the surface by streptococcal species proceeded according to the same pattern as observed for cleaned enamel surfaces in the mouth [Theilade et al., 1982; Nyvad and Kilian, 1987; Frandsen et al., 1991]. This supported previous observations by Svanberg and Loesche [1977, 1978] that resistance against colonization of teeth by mutants streptococci is a feature of the normal oral microflora.

*Actinomyces naeslundii* genospecies 1 and 2 are dominant parts of the dental plaque microflora and have overlapping substrate requirements with mutants streptococci, and streptococci from the *S. oralis* group, ‘oralis streptococci’. Therefore *A. naeslundii* genospecies are, like the other streptococci, competing with mutants streptococci. Due to their presumed role in controlling mutants streptococci, the present experiment is aimed to investigate *A. naeslundii* genospecies in dental plaque before and after intensive chlorhexidine treatment.

**Materials and Methods**

**Participants and Treatments**

After written informed consent 15 students in social sciences, between 18 and 26 years of age, participated in the study. In each subject two dental fissures were selected that contained high levels of mutants streptococci, that is more that 40% of the total cultivable flora. These fissures were treated with 40% chlorhexidine varnish [Schaeken et al., 1989]. Prior to application of the varnish, the fissures were isolated with cotton rolls and dried with compressed air. In a previous study we found that a short contact time of the varnish with the tooth surface was sufficient for effective suppression of mutants streptococci in approxi­mate areas [Schaeken et al., 1991b]. In this experiment we therefore removed the varnish 15 min after application with a dental explorer.

**Sample Collection and Bacterial Procedures**

Plaque samples from the experimental sites were collected before, and 1, 2 and 3 months after varnish application. Plaques from the two selected fissures were pooled. Prior to plaque sampling, adherent saliva on the teeth was removed by water spray. Plaque was collected using a needle (12 mm×0.4 mm) that fitted in a needle holder. The plaque was transferred into a vial containing 1 ml of isotonic saline and processed within 1 h.

The samples were homogenized by ultrasonic dispersion for 20 s at 0°C using a Kontes cell disrupter (type 9110001, Kontes, Vineland, N.J., USA). The suspensions were serially diluted 10-fold and plated onto blood agar, TSY20B agar [Schaeken et al., 1986a] and CNAC-20 agar [Ellen and Balcerzak-Raczkowski, 1975]. All plates were incubated at 37°C in an atmosphere of 91% N₂, 5% CO₂ and 4% H₂ for 5 days.

Total cultivable flora and oralis streptococci were counted on blood agar. Species of the oralis group including *S. sanguis*, *S. oralis* and *S. gordonii* [Kilian et al., 1989] cannot be distinguished by colonial morphology and were counted together. TSY20B served to enumerate mutants streptococci, and *A. naeslundii* genospecies were counted on blood agar and on CNAC-20 agar.

**Characterization of the Actinomyces Populations before and after Chlorhexidine Treatment**

The effect of chlorhexidine application on *A. naeslundii* genospecies [Johnson et al., 1990] was studied in plaque samples from 8 subjects that were taken at baseline, and at 1 month after treatment. From randomly selected colonies on the blood agar plates *Actinomyces*-like organisms were isolated. Pure cultures of 85 *Actinomyces* strains isolated at baseline and 106 *Actinomyces* strains isolated after 1 month were kept in skimmed milk at −80°C. All isolated strains were identified with the API 20A system (BioMérieux, Marcy-l’Etoile, France) and the API PLUS computer program. Additionally, the Minitek system (BBL, Cockeysville, Md., USA) was used to test the presence of nitrate and nitrite reductase, the hydrolysis of esculin, and urase activity. Acidic end products of glucose fermentation were determined by means of isothiocyanophoresis. To this end the strains were cultured under anaerobic conditions for 48 h in Trypticase Soy broth (BBL) supplemented with 5% glucose. Catalase activity was tested by emul­sifying a colony in a drop of H₂O₂ on a glass slide. Finally all strains isolated on blood agar were tested for growth on CNAC-20 agar.

**Statistical Procedures**

The bacteriological counts were log-transformed prior to statistical analyses, so that the variances would be normalized. The data were analyzed using ANOVA, and contrast (t) tests were used to evaluate differences between treatments and sampling occasions. Recoveries of *A. naeslundii* on CNAC-20 agar were analyzed with the chi-square test.

**Results**

One month after varnish application no significant differences were found in total viable counts of plaque samples and counts of oralis streptococci compared with baseline values. However, a significant increase was observed in *A. naeslundii* counts (p<0.01), while the number of mutants streptococci had significantly decreased (p<0.01) after chlorhexidine varnish treatment (fig. 1).

The characterization of the *A. naeslundii* populations before and after chlorhexidine treatment is given in table I. The percentage *A. naeslundii* genospecies of total counts on blood agar was 33% at baseline, and 61% 1 month after treatment (p<0.05). At baseline 37 strains were classified as *A. naeslundii* genospecies 1, and 48 as *A. naeslundii* genospecies 2 (formerly called *A. viscosus*), and 1 month after chlorhexidine treatment 45 strains were classified as *A. naeslundii* genospecies 1 and 61 as *A. naeslundii* genospecies 2.

At baseline 8 out of 29 (28%) *A. naeslundii* genospecies 1 strain was catalase-positive. In the samples taken at 1 month
after chlorhexidine varnish application the number of cata-
lase-positive \textit{A. naeslundii} genospecies 1 strain was sig-
ificantly (p < 0.05) lower i.e. 2 out of 45, i.e. 4%.

Only 64\% of all \textit{A. naeslundii} isolates grew on CNAC-20
agar. Growth on CNAC-20 after chlorhexidine treatment
(72\%) was significantly better than before (72 vs. 49\%;
p < 0.01; chi square). \textit{A. naeslundii} genotype 2 strains grew
better on CNAC-20 than did \textit{A. naeslundii} genotype I strain
(72 vs. 50\%, p < 0.01; chi square).

\textbf{Discussion}

After a single varnish treatment long-term selective sup-
pression of mutans streptococci was observed, which con-
firmed previous findings [Sandham et al., 1988; Schaeken
et al., 1989, 1991a, b]. A contact time between varnish and
tooth surface of only 15 min was sufficient to achieve this
long-term suppression. From earlier experiments it is
known that the suppression of oralis streptococci is of very
short duration and that these organisms recover within 1 or 2
days [Schaeken et al., 1986b, 1994]. \textit{A. naeslundii} geno-
types need about 1 week for complete recovery [Schaeken
et al., 1986b, 1989].

In this study we found that the \textit{A. naeslundii} counts were
significantly higher after the chlorhexidine varnish applica-
tion than before. Similar results were found after 1% chlor-
hexidine rinses in primates [Emilson et al., 1981] and after
chlorhexidine varnish treatment of root surfaces in peri-
dontal patients [Schaeken et al., 1991a], and in patients wear-
ning overdentures [Keltjens et al., 1992] who used chlorhexi-
dine gel daily. In contrast, Fure and Emilson [1990] reported
no increase in \textit{Actinomyces} levels after combined chlorhexi-
dine gel and chlorhexidine varnish treatments. In their study
the \textit{Actinomyces} population was back to pretreatment level
after 1 month. High levels of \textit{Actinomyces} may be consid-
ered a favorable condition as these organisms are associated
with sound tooth surfaces [Meiers et al., 1982; Brown et al.,
1986; Fure et al., 1987; Keltjens et al., 1987].

Since \textit{A. naeslundii} are involved in the resistance of the
microflora against colonization by mutans streptococci, it

\begin{table}
\centering
\begin{tabular}{lcc}
\hline
& Before & After CHX \\
\hline
\multicolumn{3}{l}{\textit{A. naeslundii} genospecies 1} \\
Catalase-positive & 8 & 2 \\
Catalase-negative & 29 & 43 \\
Total & 37 & 45 \\
\hline
\multicolumn{3}{l}{\textit{A. naeslundii} genospecies 2} \\
Catalase-positive & 48 & 60 \\
Catalase-negative & 0 & 1 \\
Total & 48 & 61 \\
\hline
Total number of isolates & 85 & 106 \\
\hline
\end{tabular}
\caption{\textit{Actinomyces} isolates before and 1 month after chlorhexi-
dine varnish (CHX) application}
\end{table}
was of interest to know whether chlorhexidine varnish treatment would induce profound changes of A. naeslundii genospecies populations. The observations fail to demonstrate such changes because the distribution of genospecies 1 and 2 was almost the same before and after chlorhexidine treatment. This supports the idea that following intensive chlorhexidine treatment, the tooth surface is sequentially repopulated by indigenous oral microflora according to the sensitivities to chlorhexidine. Within A. naeslundii genospecies 1 a shift towards catalase-negative biotypes was observed. It is difficult to speculate on the ecological significance of this phenomenon. Further, it is not known whether or not the shifts observed here are beyond normal fluctuations in the composition of the oral flora.

The recovery of A. naeslundii on CN agar was in accordance with our earlier observations, but lower than that of reference strains [Ellen and Balcerzak-Raczkowski, 1975].

We may speculate on the role of A. naeslundii in counteracting the recolonization of the chlorhexidine-treated surfaces by S. mutans. To begin with, we know relatively little of the ecological determinants of A. naeslundii. For instance, oralis and mitis streptococci, but not A. naeslundii accumulate when dental plaque is enriched on human saliva [de Jong and van der Hoeven, 1987; van der Hoeven et al., 1989]. This fits in with the early colonization of teeth by these streptococci, but fails to explain the emergence of A. naeslundii in early plaque. On the other hand, human saliva can support carbohydrate-limited growth of A. naeslundii in pure culture. With respect to the interactions between S. mutans and A. naeslundii we assume that, for most of the time, competition for free sugars is a major determinant of the population sizes of these organisms in dental plaque. This is so because both organisms depend upon carbohydrates for growth [Buchanan and Pine, 1967; Carlsson, 1970]. It is likely that at in-between meal periods, release of complex-bound sugars is the rate-limiting step in the supply of free sugar. Although A. naeslundii lose competition with S. mutans for free sugars anaerobically [van der Hoeven and de Jong, 1984; van der Hoeven and Gottschal, 1989], the organism derives competitive advantage in the presence of oxygen by increasing its cell yield [de Jong et al., 1988; van der Hoeven and van den Kieboom, 1990]. Further, A. naeslundii is less sensitive to growth inhibition by oxygen than S. mutans [van der Hoeven and Gottschal, 1989].

Collectively, the results of this study strongly suggest that the indigenous populations of A. naeslundii genospecies are involved in controlling the return of mutans streptococci after chlorhexidine treatments.

References


