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Effect of chlorhexidine mouth rinse on *Streptococci* counts of tooth-tissue-borne palatal expander biofilm

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Structured Abstract

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Objectives – To assess total *Streptococci* (TS) counts and biofilm mass over tooth-tissue-borne palatal expander (TTBPE), as well as the effect of chlorhexidine (CHX) mouth rinse on these variables.

Design – A cross-sectional study design employed clinical procedures and laboratorial techniques.

Setting and Sample Population – Patients who had TTBPE removal indicated were divided into two groups: a CHX group (n = 26) in which three times a day of 0.2% CHX digluconate mouth rinses were prescribed 7 days before TTBPE removal; and a control (CON) group (n = 25) in which no antimicrobial treatment was applied.

Experimental Variable – 'Gender', 'Age', and 'TTBPE wear time' were recorded. After TTBPE removal, biofilm mass was determined by the difference between (TTBPE + biofilm) and (TTBPE only) masses. TS counts were determined by biofilm suspension followed by progressive dilutions and culture on Mitis Salivarius agar with incubation at 37°C for 72 h.

Outcome Measure – Biofilm mass (mg) and Colony Forming Units of TS/mg of biofilm (CFU-TS/mg) were calculated.

Results – Total *Streptococci* mean values in CHX (6.77×10^6CFU-TS/mg) were statistically lower ($p < 0.01$) than those in CON (3.82×10^7CFU-TS/mg), but there was no statistical difference ($p > 0.05$) between CHX (168.88 mg) and CON (182.04 mg) masses nor statistical correlation ($p > 0.05$) between biofilm mass and CFU-TS/mg in the two groups.

Conclusion – Chlorhexidine reduces the TS counts in TTBPE, but has no effect on biofilm mass.

Key words: chlorhexidine; microbiology; orthodontics; palatal expander

Introduction

The tooth-tissue-borne palatal expander (TTBPE) presents a larger orthopedic effect (1) and less reduced alveolar bone crest level of supporting teeth (2) than the tooth-borne expander. However, in a healthy oral cavity, microbiota coexists in a state of balance with their host, and the

placement of orthodontic appliances unbalances this environment and disease may result (3). The most common side effects of fixed orthodontic appliances, namely decalcification (4) and periodontal disease (5) are caused by bacteria. Both design and surface of orthodontic accessories and bonding material may influence biofilm formation (6–10).

Considering TTBPE, the importance of decalcification and periodontal disease risks are outweighed by 50% prevalence of streptococcal bacteremia following the TTBPE removal procedure (11), which is much higher than the 10% (12) and 7.5% (13) bacteremia prevalence following banding and 6.6% (14) following debanding. This greater prevalence could be explained not only by the presence of four bands in TTBPE, but also by the presence of a thick biofilm over the acrylic pad. The microbial composition of biofilms on these appliances is still unknown (11). Moreover, as bacteremia is the result of bacterial presence and soft-tissue aggression (12), TTBPE acrylic pad microorganisms have mucosal inflammation – reported since the first TTBPE studies in pigs and humans (15) – as a suitable access to the bloodstream.

Thus, reduction in the quantity of TTBPE bacteria would not only diminish the risk of caries and periodontal disease, but also the occurrence of bacteremia. Chlorhexidine (CHX) is known for its specificity, efficacy, substantivity, safety and stability (16), and its use by orthodontic patients has positive clinical (17) and microbiological (18) results. Moreover, Erverdi et al. (19), while assessing the application of CHX mouthwash prior to orthodontic banding and debanding noticed a three-fold reduction in bacteremia.

The aim of this study was to assess the quantity of total *Streptococci* (TS), biofilm mass, and their correlation in TTBPE, as well as the effect of CHX on these variables.

Materials and methods

Subjects

This research project was approved by the Pontifical Catholic University of Paraná Research Ethics Committee (Of. 011/06). Sixty-four patients of the Dental Master's program on whom removal of their TTBPE was indicated as a part of their treatment plan were examined. Only healthy patients who presented no heart or valve diseases, immunosuppression, diabetes, and antibiotic use 3 months before TTBPE removal or regular antiseptic use were selected. Factors such as gender, age, and TTBPE wear time were recorded for statistical analyses.

Each TTBPE had a different design (related to patient's palate anatomy), quantity of activations (related to treatment planning), and operator. Patients were instructed not to change their oral hygiene habits. These potential confounding factors were solved by randomization.

Patients were randomly divided into two groups: a CHX group (n = 34; age 10 year 3 months ± 2 year 3 months), in which 0.2% CHX digluconate was prescribed and a control group (CON, n = 30; age 9 year 4 months ± 1 year 4 months), in which no antimicrobial treatment was prescribed. Informed consent was obtained from at least one guardian of all patients.

During the research progress, five CON group patients began antiseptic rinsing before the TTBPE removal session and eight patients in the CHX group did not follow the rinsing protocol (described below), one patient because of mucosal desquamation and seven because of negligence. The final sample distribution to 51 subjects is shown in Table 1.

Table 1. **Demographics of sample**

	Chlorhexidine	Control
n	26	25
Gender		
Male	11	8
Female	15	17
Age (years/months)		
Mean	10 year 3 months	9 year 4 months
SD	2 year 3 months	1 year 4 months
Median	9 year 5 months	9 year 6 months
Minimum	7 year	7 year 5 months
Maximum	15 year 10 months	11 year 6 months
Expander wear time (days)		
Mean	209.35	200.28
SD	86.06	106.30
Median	178.50	147.00
Minimum	119.00	77.00
Maximum	405.00	421.00

Chlorhexidine rinse protocol

The protocol consisted of three daily mouth rinses with 5 ml of 0.2% CHX digluconate 7 days before TTBPE removal. A 30-s rinsing period was recommended for each mouth rinse, as a result of a pilot study that tested 30-, 45-, and 60-s mouth rinse periods in three volunteers who wore TTBPE and were of a similar age as the sample subjects, but whose TTBPE was not evaluated.

Bacteriological assessment

In both groups, TTBPE was removed by a standardized procedure avoiding any biofilm disruption, dislodgement or contamination. Subjects were instructed not to brush their teeth nor to eat 2 h before the TTBPE removal session. Only one patient from CHX group returned with yellow-brown stained teeth, but prophylaxis easily removed the stains. In both TTBPE groups, biofilm presented mineralization features and adhered firmly to the appliance.

Once removed, TTBPE mass was determined with analytical scale (Bel Mark U210A, Bel Engineering, Piracicaba, Brazil), on a sterilized aluminum foil. Next, it was immersed in 100 ml of sterile distilled water in a screw-cap bottle. This bottle was put into an ultrasound tank (Thornton T7, Thornton Inpec Eletrônica Ltda, Ribeirão Preto, Brazil) and the adhered biofilm mass was removed with continuous pulses (500 W/15 min). After biofilm removal, TTBPE was maintained in dry heat at 37°C for 48 h and weighed again. Biofilm mass was considered as the difference between TTBPE mass before and after biofilm collection.

The bottle with biofilm suspension was shaken in a vortex (AP56, Phoenix Ltda, São Paulo, Brazil) (2400 rpm/ 30 s) and opened in a class II biological security cabinet (VLFS 12, Veco do Brasil, São Paulo, Brazil). From each suspension, a 100 μl aliquot was taken and processed by serial dilutions from 10 to 100 000 times in 10-fold increments. From each serial dilution tube, duplicate 10 μl aliquots were placed on Mitis Salivarius Agar (BD, Diagnostic Systems, Sparks, MD, USA). Plates were kept in jars (Permution, Curitiba, Brazil) with 10% pCO_2 at 37°C for 72 h.

Only plates with 30–300 colonies were used for the bacterial count, as this count interval presents less experimental errors by operators. Results were expressed in Colony Forming Units of TS/ml (CFU-TS/ml) and standardized to CFU-TS/mg of biofilm. Finally, the log (CFU-TS/mg biofilm) values were also calculated.

Results

To assess the effects of CHX, group homogeneity is necessary. Gender, age, and TTBPE wear time were considered to statistically compare the CHX and CON groups. The Chi-square test demonstrated that groups were not dependent on gender ($p > 0.05$).

'Age' and 'TTBPE wear time' did not present normality by the Kolmogorov–Smirnov and Shapiro–Wilk tests ($p < 0.05$), although they presented variance homogeneity by the Levene test ($p > 0.05$). Thus, groups were compared by the non-parametric Mann–Whitney '*U*'-test which showed that there was no difference between them considering 'Age' and 'TTBPE wear time' ($p > 0.05$).

'TTBPE wear time' was assessed considering 'Gender', 'Group' and considering both factors through the two-way ANOVA. Results demonstrated that there were no difference between CHX and CON Groups ($p > 0.05$). Confounding factors, such as the TTBPE design, patients' treatment planning, and operator were randomly divided into groups, therefore any difference in biofilm mass and TS counts between groups was because of CHX mouth rinses. Descriptives of 'biofilm mass', 'CFU-TS/mg of biofilm', and 'log (CFU-TS/mg of biofilm)' are presented at Table 2 (CHX group) and Table 3 (CON group).

'Biofilm mass', 'CFU-TS/mg of biofilm', and 'log (CFU-TS/mg of biofilm)' were evaluated by the Kolmogorov–Smirnov and Shapiro–Wilk tests to verify their normality. Only the CON group's 'biofilm mass' and CHX group's 'log (CFU-TS/mg of biofilm)' presented normality ($p > 0.05$). The Levene test was used to verify the variance homogeneity of variables and showed that 'biofilm mass' and 'CFU-TS/mg of biofilm' did not present variance homogeneity ($p < 0.05$).

Because of these features, the groups were compared by the non-parametric Mann–Whitney '*U*'-test that showed that there was no statistical difference in 'biofilm mass' between groups ($p = 0.14$) and that 0.2% CHX digluconate statistically reduced 'CFU-TS/mg of biofilm' and 'log (CFU-TS/mg of biofilm)' ($p < 0.01$, Table 4). The Student's 't'-test showed the same results,

Table 2. **Chlorhexidine group descriptives of 'Biofilm Mass', 'CFU-TS/mg of Biofilm', and 'log (CFU-TS/mg of Biofilm)'**

Variable	n	Mean	SD	Median	Minimum	Maximum
Biofilm mass (mg)	26	168.8846	89.2649	145.0500	33.9000	419.4000
CFU-TS/mg of biofilm	26	6.7682×10^6	7.7680×10^6	4.1718×10^6	6.4277×10^5	3.1079×10^7
log (CFU-TS/mg of biofilm)	26	6.5653	0.5075	6.6201	5.8081	7.4925

CFU-TS, colony forming units of total *Streptococci*/mg of biofilm.

Table 3. **Control group descriptives of 'biofilm mass', 'CFU-TS/mg of biofilm', and 'log (CFU-TS/mg of biofilm)'**

Variable	n	Mean	SD	Median	Minimum	Maximum
Biofilm mass (mg)	25	182.0360	49.4263	166.3000	113.4000	296.9000
CFU-TS / mg of biofilm	25	3.8246×10^7	4.7056×10^7	1.4661×10^7	3.6541×10^6	1.5208×10^8
log (CFU-TS / mg of biofilm)	25	7.3048	0.4840	7.1662	6.5628	8.1821

CFU-TS, colony forming units of total *Streptococci*/mg of biofilm.

Table 4. **Mann–Whitney 'U'-test comparing 'biofilm mass', 'CFU-TS/mg of biofilm', and 'log (CFU-TS/mg of biofilm)' between groups**

	Mean rank			
Variable	Control (n = 25)	Chlorhexidine (n = 26)	Z	*p*
Biofilm mass	23.0000	29.1200	−1.4697	0.1416
CFU-TS/mg of biofilm	17.3462	35.0000	−4.2395	0.0000*
log (CFU-TS/mg of biofilm)	17.3462	35.0000	−4.2395	0.0000*

CFU-TS, colony forming units of total *Streptococci*/mg of biofilm.
*$p < 0.05$ means statistical difference between groups.

although it is not indicated for variables that do not present normal distribution.

Spearman test demonstrated that there was no statistical correlation between 'biofilm mass' and 'CFU-TS/mg of biofilm' in both the CON ($r = -0.1127$; $p > 0.05$) and CHX groups ($r = 0.3025$; $p > 0.05$). Furthermore, there was no correlation between 'biofilm mass' and 'log (CFU-TS/mg of biofilm)' in both the CON ($r = -0.1127$; $p > 0.05$) and CHX groups ($r = 0.3025$; $p > 0.05$).

Discussion

Every biofilm follows a series of similar developmental stages: 1) adherence of cells to a conditioned surface; 2) rapid division and growth of adherent cells; and 3) a plateau of accumulation (20). In environments that support a mixed planktonic flora, the final composition of the biofilm will reflect the outcome of bacterial succession, resulting from competition among adherent bacteria (20). Beighton and Hayday (21) assessed streptococcal growth rates on the molars of monkeys that were fed different diets in 6, 18, 24, 42 and 96 h periods and found a stable population of these bacteria between 18 and 24 h irrespective of food type. They stated that interactions with other bacteria, the ability to compete for and assimilate nutrients from the immediate environment, maintenance of energy requirements and the specific and non-specific antibacterial systems of saliva limit the natural flora's doubling time in monkeys. This assumption may be applied to TTBPE and we presume that the bacterial quantities on acrylic surfaces tend to stabilize after a certain period. Therefore, TTBPE wear time does not significantly influence the *Streptococci* counts.

As biofilms develop from single layers to multi-cell layers with intercellular matrices (20), TTBPE biofilm older than 200 days and a difficult cleaning environment should be an aggregate of live and dead cells of a stable bacteria population, as well as a large quantity of extracellular matrix.

In addition to these features, when removed, TTBPE biofilm was hardened, suggesting mineralization. To explain this phenomenon, one should understand how these biofilms are formed. Once installed, TTBPE is exposed to saliva. In the oral cavity, acquired pellicle

adsorbs to all exposed surfaces to which bacteria will adhere (20). Saliva is composed of proteins which, when immobilized on surfaces behave as mineralization initiators, presumably by binding calcium ions (22). As salivary flow promotes greater supragingival calculus formation (23) and as TTBPE is fixed for a prolonged period, biofilm mineralization may be assumed.

No studies concerning TTBPE biofilm bacteria were found and therefore, direct result comparison cannot be made. The mean quantity of TTBPE-TS in the CON group (7.30 log) of the present study was greater than that present in the dental plaque of the 96-h molar palatal grooves of monkeys that were fed a cariogenic high-sucrose diet (6.30 ± 0.49 log) (21). This also occurred in the dental plaque of maxillary molars (6.30 ± 0.72 log) and incisors (6.10 ± 0.96 log) of adult humans before a 6-month protocol of daily 0.12% CHX mouth rinses began (24). Whereas in the CON group, the quantity of TS/mg of biofilm (3.82×10^7 CFU/mg of biofilm) was far lower than that of patients with fully fixed orthodontic appliance bonding for 1 month (1.51×10^{14} CFU/mg of biofilm), and even lower than that of these same patients before appliance placement (5.20×10^7 CFU/mg of biofilm) (41). However, when comparing these results, the biofilm formation period, extracellular matrix and TTBPE cleaning difficulty must be considered.

By means of the present study protocol, CHX reduced TS counts ($p < 0.01$). This reduction of almost 1 log in the CHX group (6.57 log) in comparison with the CON (7.30 log) is very close to the reduction perceived when the quantity of TS was evaluated in the dental plaque of human molars (6.04 ± 1.00) and incisors (6.19 ± 1.10) after 6 months of daily rinses with 0.12% CHX (24). In *Streptococcus sanguis* and microcosm biofilms, CHX may diminish *Streptococci* counts by 1 log after the first administration and by 3 log in early forming biofilm (26). However, bacteria re-initiate colonization (26). Furthermore, this reduction may be jeopardized by regular carbohydrate intake (25). One- and five-minute periods of mouthwash simulators do not alter biofilm viability; this is only achieved by a 1-h exposure to CHX (27).

The biofilm masses of the CHX and CON groups were comparable and this is in accordance with the fact that dead microorganisms are only removed by mechanical plaque removal (18). The present results suggest that the contiguous palatal mucosa/TTBPE acrylic pad interface hinders mechanical removal by mouth rinses of TS, which were killed by CHX.

The CHX mode of action is most likely related to the extensive intracellular damage rather than cell lysis (28). The pharmacological features of CHX and TTBPE biofilm complexity explain why greater TS reduction could not be accomplished. CHX retention is diminished at a more acidic pH (29). Vroom et al. (30) demonstrated that biofilm pH is more acidic at greater depths. Moreover, it is known that CHX is much less effective for killing mutans *Streptococci* and *Lactobacilli* in a biofilm than in the laboratory, probably because it does not reach the microorganisms located at a deeper level (31). Nevertheless, as previously mentioned, it is likely that TTBPE biofilm presents a considerable quantity of mineral ions. Positive ions like calcium may occupy the same binding sites as CHX and limit its retention (32–34).

Bacteria that are members of a biofilm community are generally less susceptible even to antibiotics than are their planktonic counterparts, probably because of: 1) modified nutrient environments and suppression of growth rate; 2) a glycocalyx that constitutes a physical barrier to environmental fluctuations; or 3) the development of biofilm/attachment-specific phenotypes, which have a modified susceptibility toward antimicrobials (35–37). More related to CHX, its diffusion in biofilm is limited, as it only reaches the deeper layers after 5 min (38). This demonstrates that one cannot expect a greater reduction in TS counts with the exclusive use of CHX mouth rinses even when used with greater frequency or for a longer period.

In both groups, there was no statistical correlation between biofilm mass and quantity of TS ($p > 0.05$) and this demonstrates that TTBPE biofilm is a complex structure and needs further studies. It is expected that the biofilms of these appliances such as dental plaque consist of cells, extracellular matrix, empty spaces and substratum (39). A better understanding of their three-dimensional structures is essential to develop more efficient therapeutic procedures.

More relevant than the risk of caries and periodontal disease development, TTBPE removal presents 50% prevalence of bacteremia (11). It is not yet possible to know whether the quantity of TTBPE-TS reduction achieved in the present research may prevent the

occurrence of bacteremia because up to the present moment, there is no understanding about the correlation between bacteremia and the cellular density of oral environment microbiota. With regard to this issue, the American Heart Association guidelines (40) are not clear, for they do not include TTBPE removal as a procedure with a high risk for bacteremia.

It is expected that the present study results may be a potential base for developing protocols that minimize or hinder not only the risk of caries and periodontal disease, but also bacteremia. This information will be very useful in the treatment of moderate and high-risk infective endocarditis subjects, who need an enhanced orthopedic effect on their rapid maxillary expansion.

Conclusions

On TTBPE, CHX reduces the quantity of biofilm TS, but it has no effect either on biofilm mass or the relationship between the quantity of TS and biofilm mass.

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