Evaluation of the Contamination and Disinfection Methods of Toothbrushes Used by 24- to 48-month-old Children

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ABSTRACT

Purpose: The purpose of this study was to evaluate: (1) in vivo the contamination by mutans streptococci (MS) of toothbrushes after use on 52 children (24-48 months old) by a single dentist; (2) in vivo the efficacy of 3 solutions (Periogard, Brushtox, and a Cosmocil CQâ and Myacide pharma BPâ-based experimental solution) in the disinfection of these toothbrushes through a randomized clinical trial; and (3) in vitro the antimicrobial activity of the solutions by the agar diffusion test using 15 microbial strains.

Methods: In the in vivo trial, children were randomly assigned to 1 of 4 groups (N=13) and a 4-stage changeover system was used with a 1-week interval between each stage. Solutions were used by a different group of children in each stage. Children were submitted to a 1-minute brushing (without toothpaste) performed by a single professional, followed by random spraying of the test solutions and microbiological analysis.

Results: Brushtox, Periogard, and the experimental solution reduced/prevented the formation of MS colonies/biofilms on the toothbrush bristles compared to the control (sterile tap water; $P<.001$). Periogard and the experimental solution showed significantly greater reduction of colonies/biofilms compared to Brushtox ($P<.01$). In the in vitro experiment, Periogard exhibited the greatest inhibition halo average, followed by the experimental solution, Brushtox, and sterile tap water ($P<.05$).

Conclusions: After a single brushing, severe contamination by mutans streptococci colonies/biofilms was observed on all toothbrushes sprayed with sterile tap water (control). Although Brushtox presented better results than sterile tap water, Periogard and the experimental solution showed greater efficacy against formation of MS colonies/biofilms on the toothbrush bristles and exhibited larger microbial growth inhibition halos. (J Dent Child 2006;73:152-158)

KEYWORDS: TOOTHBRUSHES, MUTANS STREPTOCOCCI, CHLORHEXIDINE

Toothbrushes are manufactured free of microorganisms.[1,2] After a single use for periods varying from 30 seconds to 4 minutes,[3,4] however, toothbrushes may become contaminated by a wide array of bacteria,[2-10] viruses,[11,12] yeasts, and fungi,[13,14] which are present both in the oral cavity and in the external environment.[15]

Microorganisms can remain viable on toothbrush bristles for periods ranging from 24 hours to 7 days.[5,13,16,17] Therefore, the routine use of contaminated toothbrushes might contribute to disseminate microorganisms within the oral cavity of a same person or between different individuals.[16,17] Occasionally, toothbrushes belonging to different members of the same family may be in direct contact when stored in the same toothbrush holder or put together in bathroom drawers or cabinets.[18] In addition, salivary contact is hardly

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controlled among children staying in day-care centers, kindergartens, and other institutions that shelter children at an early age,[6] where toothbrushes can be inadvertently exchanged or shared.

Modern dentistry strongly emphasizes prevention and biosecurity regarding how toothbrushes should be appropriately stored, disinfected, and changed at regular intervals. Nevertheless, few in vivo studies have investigated the microbial contamination of toothbrush bristles after use and the most effective disinfection methods.[2,4,9,10,15,19,20]

This study's purpose was to evaluate:

1. in vivo the contamination of toothbrushes by mutans streptococci (MS) after use on children at an early age by a single professional;
2. in vivo the efficacy of Periogard, Brushtox, and an experimental solution in the disinfection of these toothbrushes through a randomized clinical trial (microbial culture and scanning electron microscopy [SEM]); and
3. in vitro the antimicrobial activity of these 3 solutions by the agar diffusion test.

METHODS

RANDOMIZED CLINICAL TRIAL

This study was approved by the Ethics in Research Committee of the Faculty of Dentistry of the University of São Paulo, São Paulo, Brazil, and written informed consent was obtained from all parents or guardians.

Fifty-two children of both sexes (29 boys and 23 girls), 24 to 48 months old (mean age=39 months), were selected from a day-care center according to the following inclusion criteria. The participants should:

1. have complete primary dentition;
2. not be under dental treatment;
3. not be under therapy with antibiotics or antiseptic mouthrinses for at least 3 months; and
4. present MS in saliva, as detected in SB_{20} culture medium prepared according to Davey and Rogers[21] and modified by replacement of sucrose with sugarcane.[2,4]

The baseline MS level in the saliva of all 52 children before the toothbrushing experiment ranged from 20 to 3,000,000 cfu/mL.

The following solutions were evaluated:

1. Periogard (0.12% chlorhexidine solution; Colgate Palmolive, Kolynos do Brazil Ltd, São Paulo, Brazil);
2. Brushtox antiseptic toothbrush cleaner spray (activated ethanol, 35%-40% volume to volume (v/v) with a biocide, Dentox Limited, Warwickshire, England);
3. an experimental solution (composition: Cosmocil CQ[å] [1% polyaminopropyl biguanide], Myacide pharma BP[å] [0.1% bronopol], ethylenediaminetetraacetic acid [EDTA], propyleneglycol, polyvinylpirrolidone K30, ethanol [96°], 0.5 M sodium hydroxide solution, distilled water, and blue no.1 dye; and
4. sterile tap water (control group).

Brushtox spray was maintained in its original receptacle, while Periogard, the experimental solution, and sterile tap water were placed in individual plastic trigger-spray bottles (Elyplast; São José dos Campos, São Paulo, Brazil). To perform a blind evaluation of the solutions, the bottles were covered with aluminum paper and codified.

Using a table of random numbers, 52 children were randomly chosen, thus forming 4 groups of 13 children each. A 4-stage changeover system was used, with a 1-week interval between each stage. All 4 solutions were used in all stages, but each solution was used by a different group of children in each phase of the study to minimize the occurrence of variables that could interfere with the results. In each stage, the children were submitted to a 1-minute brushing—performed at the day-care center by a single dentist (a postgraduate dental student)—without dentifrice and using new toothbrushes taken directly from their original packages (Colgate Baby— Barney, Colgate/Palmolive, Kolynos do Brazil Ltd, São Bernardo do Campo, São Paulo, Brazil).

After toothbrushing, the bristles were rinsed and excess water was removed. The toothbrushes were held in a vertical position, and the solutions were sprayed 6 times onto the bristles at a distance of 5 cm (approximately 0.6 mL of solution per toothbrush) in different areas: (1) right side; (2) left side; (3) top; (4) bottom; (5) front; and (6) back of the toothbrush head. Excess antimicrobial solution was removed from the bristles by gently hitting the toothbrush against the sink.

Thereafter, the toothbrushes were maintained in a closed custom container to avoid contact between them and were kept at room temperature over 4 hours to simulate the interval between brushings.[2,4]

To investigate whether the toothbrushes presented contamination deriving from manufacturing and packaging processes, 5 unused toothbrushes (additional control) were taken from their original packages and submitted to microbiological processing.

All examiners were blinded to the group being examined microbiologically or by SEM.

MICROBIOLOGICAL PROCEDURES

After 4 hours simulating the interval between brushings, the toothbrushes of each group were individually and vertically placed into 25x150 mm test tubes containing 10.0 mL CaSa B (Bacitracin Sucrose Broth—selective enrichment broth prepared by the modification of Jensen and Brattall,[23] medium specific for mutans streptococci without trypan blue) for 3 to 4 days at 37°C. Care was taken to avoid contact between the bristles and test tube walls. The toothbrushes were withdrawn and rinsed in the broth with gentle shaking to remove planktonic microbiota, leaving sessile bacteria adhered as "spike" or "mushroom-like" colony/biofilms. The toothbrush bristles were carefully analyzed on all sides, and sessile mutans streptococci colonies/biofilms, based on colony morphology, were counted under aseptic conditions with a stereomicroscope (Nikon, Tokyo, Japan) with reflected light.

The number of MS colonies/biofilms on the bristles' surfaces was expressed according to a ranked scale as follows:

1. score 1=1 to 50 colonies/biofilms;
2. score 2=51 to 100 colonies/biofilms;
3. score 3=over 100 colonies/biofilms (intense bacterial growth, with confluent colonies, not allowing an accurate counting of the number of colonies/biofilms);
4. score 0=no colonies/biofilms were detected, indicating absence of microorganisms on bristle surface.

Confirmation that the adhered microorganisms were MS was obtained by a sequence of steps:

1. Four to 5 colonies/biofilms representative of bacterial development were collected from the bristles of 3 to 4 toothbrushes in each group and transferred to tubes containing 2.0 mL of phosphate-buffered solution and glass beads.
2. The colonies were vortexed for 2 minutes.
3. The resulting suspension was seeded on SB_{20} agar (tryptone soy yeast agar plus 20% sucrose and 0.2 U/mL Bacitracin; Sigma, Saint Louis, Mo) and incubated in microaerophilia at 37°C for 72 hours.
4. The growth of colonies/biofilms was verified after the incubation period.
5. The following tests were performed for biochemical identification:[24]
   a. fermentation of mannitol, sorbitol, raffinose and melibiose;
   b. hydrolysis of arginine and sculin;
   c. production of H_2O_2; and
   d. sensitivity to 2.0 IU bacitracin.

The microbiological results were submitted to statistical analysis by Friedman's nonparametric test at the 5% significance level, using 8.1 GMC statistical software package (Dr. Campos, Faculty of Dentistry of Ribeirão Preto, University of São Paulo, São Paulo, Brazil).

SCANNING ELECTRON MICROSCOPY (SEM)

After microbiological processing, 4 representative toothbrushes of each group were fixed in 4% glutaraldehyde in cacodylate buffer pH 7.4 at 37°C. Two bristle tufts of each toothbrush were: (1) removed; (2) post-fixed in 1% osmium tetroxide; (3) dehydrated in ascending ethanol grades; and (4) critical-point dried with liquid carbon dioxide.

Subsequently, 8 bristles of these tufts were:

1. separated;
2. mounted on stubs;
3. sputter-coated with gold; and
4. examined in a Zeiss (DSM 940A, Jena, Germany) SEM at 15 kV.

IN VITRO EXPERIMENT

The antimicrobial activities of Brushtox, Periogard, and experimental solution was assessed by the agar diffusion test, as described by Groove and Randall.[25] The following microbial strains were used:

1. *Micrococcus luteus* (ATCC 9341);
2. *Staphylococcus aureus* (ATCC 6538, ATCC 25923, penicilinase positive field strain and penicilinase negative field strain);
3. *Candida albicans* (ATCC 1023 and field strain–saliva);
4. *Candida tropicalis* (field strain–saliva);
5. *Escherichia coli* (ATCC 10538);
6. *Pseudomonas aeruginosa* (ATCC 2327);
7. *Enterococcus faecalis* (ATCC 10541);
8. *Streptococcus mutans* (NTC 1023, ATCC 25175 and field strain–saliva); and
9. *Streptococcus sobrinus* (field strain–saliva).

The agar diffusion test was prepared in triplicate by the agar-well method (double-layer technique) using the Brain Heart Infusion (BHI, Difco Laboratories, Detroit, Mich) culture medium for streptococci and enterococci, and the Mueller Hinton (MH; Difco) culture medium for the other microorganisms. The base layer was obtained by pouring 10mL of BHI or MH culture medium at 50°C in 20 x100 mm Petri plates. After solidification of the base layer, the seed layer (5 mL of BHI or MH culture medium at 50°C) was added, with 10^8 colonies/biofilms per mL of original inoculum (adjusted to a 2.0 McFarland standard) for yeasts or 10^6 colonies/biofilms per mL of original inoculum (adjusted to a 0.5 McFarland standard) for the other tested microorganisms.

After solidification, 4 equidistant perforations (wells) were made in each plate. The wells had 5 mm in diameter and were deep enough to reach both base and seed layers. Twenty microliters of each solution were poured into each well and incubated for 2 hours at room temperature. Thereafter, the MH plates were incubated in anaerobiosis and the BHI plates were incubated in microaerophilia (candle jar system) at 37°C for approximately 24 hours. The microbial growth inhibition halos were measured in millimeters using a rule under reflected light.

Data were analyzed statistically by analysis of variance and Tukey's test at the 5% significance level, using GraphPad Prism 4.0 for Windows version 4.0 statistical software package (GraphPad Software Inc., San Diego, Calif.).

RESULTS

RANDOMIZED CLINICAL TRIAL: MICROBIOLOGICAL RESULTS

From 52 children initially enrolled in this study, only 45 (87%) participated in all 4 stages of the randomized clinical trial. Table 1 shows the number of cases per score attributed according to the number of MS colonies/biofilms formed on the toothbrush bristles after brushing and spraying with the tested solutions.

MS colonies/biofilms were detected on the bristles of all (100%) toothbrushes in the control group (sprayed with sterile tap water), with a strong predominance of score 3. The number of colonies/biofilms ranged from 2 to uncount able (Figures 1a and 1b). After use of Brushtox antiseptic cleaner spray, MS colonies/biofilms were observed in 12

Table 1. Number and Percentage of Cases Per Score Attributed According to the Number of Mutans Streptococci Colonies/Biofilms Formed on the Bristles of the Children's Toothbrushes After Brushing and Spraying With the Tested Solutions

Score	Sterile tap water	Periogard	Experimental solution	Brushtox
0	0	45 (100%)	45 (100%)	33 (73.3%)
1	2 (4.4%)	0	0	4
2	2 (4.4%)	0	0	8 (17.8%)
3	41 (91.2%)	0	0	0 (8.9%)

From the 52 children initially enrolled in this study, only 45 participated in all four stages of the randomized clinical trial.

toothbrushes (27%), but none of them was scored 3. The number of colonies/biofilms ranged from 4 to 100 (Figure 2a). On the other hand, when Periogard and the experimental solution were used (Figure 3a), no colonies/biofilms were observed in 100% of the cases (ie, all toothbrushes in this group scored 0).

Based on these microbiological results, it may be inferred that Brushtox, Periogard, and the experimental solution reduced/prevented the formation of colonies/biofilms on the toothbrush bristles' surfaces, as all these solutions differed statistically from the control solution (sterile tap water; $P<.001$). Periogard and the experimental solution were statistically similar to each other ($P>.05$) and had better results as compared to Brushtox ($P<.01$).

There was no bacterial contamination on the 5 unused toothbrushes after incubation at 37°C for 20 days.

SCANNING ELECTRON MICROSCOPY

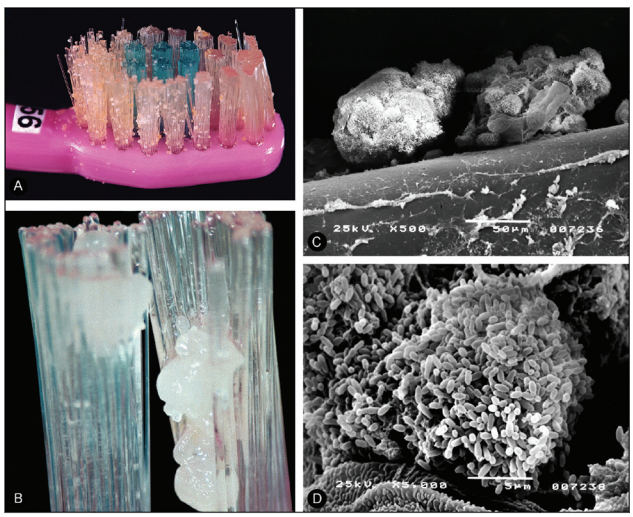

Figure 1. Sterile tap water: A and B— Presence of a great number of mutans streptococci colonies/biofilms on the toothbrush bristles, after microbial culture. C and D— SEM micrograph showing the formation of MS colonies/biofilms (X500 and X5000 magnification)

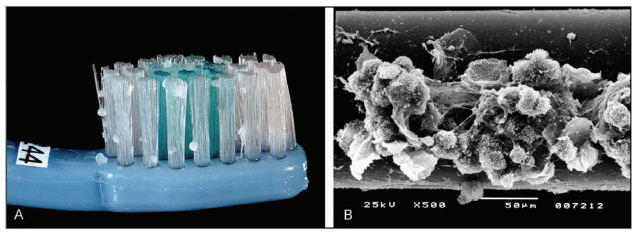

Figure 2. Brushtox: A— Presence of a small number of mutans streptococci colonies/biofilms on the tooth-brush bristles, after microbial culture. B— SEM micro-graph showing the formation of MS colonies/biofilms, after microbial culture. (X500 magnification).

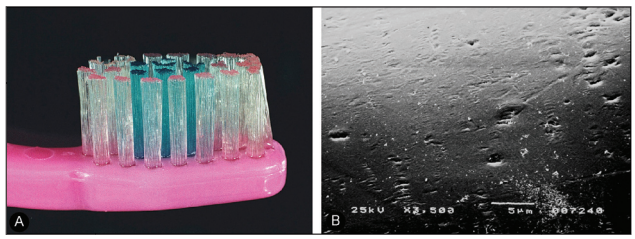

Figure 3. A— Toothbrush representative of the lack of formation of mutans streptococci colonies/biofilms on the bristles, after spraying with Periogard or experimen tal solution. B— SEM micrograph representative of tooth brush bristles sprayed with Periogard or experimental solution, showing absence of microorganisms (X3500 magnification).

In all groups, when the microbiological culture was positive via detection of colonies/biofilms under stereomicroscopy, mutans streptococci bacterial biofilm was also observed adhered to the toothbrush bristles upon SEM analysis (Figures 1c and 2b). When colonies/biofilms were not observed upon analysis on a stereomicroscope, no microorganisms or only sparse microorganisms were observed on SEM examination (Figure 3b).

IN VITRO EXPERIMENT

Table 2 shows the antimicrobial activity of the tested solutions, evaluated by the agar diffusion test using 15 target microbial strains.

All solutions differed statistically from each other ($P<.05$). Periogard presented the greatest inhibition halo average (19.3±3.2 mm), followed by the experimental solution (12.4±3.14 mm), Brushtox (7.8±6.3 mm), and sterile tap water (0 mm).

DISCUSSION

In this study, there was 100% of contamination by MS on toothbrush bristles after a single 1-minute brushing followed by spraying with sterile tap water (control). These findings are in agreement with those of other studies.[2,4,5,9,16,26]

Disinfection of a new toothbrush should be initiated just after the first brushing to prevent bacterial biofilm formation on bristles' surfaces.[27] Thereafter, disinfection should be done daily until the toothbrush is replaced, which should be done every 3 or 4 months, according to the American

Table 2. Antimicrobial Activity of Sterile Tap Water, Brushtox, Periogard, and Experimental Solution Against 15 Microbial Strains

Microorganism	Sterile tap water	Periogard	Experimental Solution	Brushtox
Micrococcus luteus	0.0	20.5	19.5	26.5
Staphylococcus aureus	0.0	19.0	14.0	10.0
Staphylococcus aureus	0.0	19.5	15.0	8.5
Staphylococcus aureus	0.0	19.0	17.0	7.0
Staphylococcus aureus	0.0	13.0	11,0	10.5
Candida albicans	0.0	20.0	13.5	0.0
Candida albicans	0.0	19.0	12.0	0.0
Candida tropicalis	0.0	19.5	11.5	0.0
Escherichia coli	0.0	16.0	13.0	8.5
Pseudomonas aeruginosa	0.0	15.5	13.0	7.0
Enterococcus faecalis	0.0	24.0	12.0	8.0
Streptococcus mutans	0.0	25.0	<7.0	9.5
Streptococcus mutans	0.0	22.0	11.0	7.5
Streptococcus mutans	0.0	21.5	10.0	7.0
Streptococcus sobrinus	0.0	15.5	<7.0	<7.0

Measurements of the inhibition halos in millimeters (agar diffusion test).

Dental Association.[28]

Several studies have suggested the need for toothbrush disinfection to reduce the number of microorganisms on the bristles using different methods, including:

1. UV radiation[29];
2. microwave irradiation[29];
3. boiling water[29]; and
4. chemical agents, such as
   a. Listerine[15];
   b. Plax[15];
   c. Cepacol[15]; and
   d. chlorhexidine.[2]

In addition, some authors have attempted to incorporate antimicrobial agents, such as silver,[7] chlorhexidine,[8,29] and others[9] to the toothbrush bristles as a coating layer during manufacturing process.

In this study, Periogard spray proved 100% efficacious for toothbrush disinfection, as no MS colonies/biofilms were observed in this group. These findings are consistent with those of Nelson-Filho et al,[2] who observed an absence of MS growth on the bristles of toothbrushes soaked in 0.12% chlorhexidine gluconate.

Microbial growth inhibition halo average of Periogard (19.3±3.2 mm), as detected by the agar diffusion test, was larger than those of the other solutions, with this difference being significant statistically. This outcome is consistent with the conclusions of Moshrefi,[30] who states that chlorhexidine still is the "gold standard" antimicrobial agent when comparing with other solutions used for dental biofilm control.

The experimental solution tested in this study uses Cosmocil CQ[â] and Myacide pharma BP[â] as active components. Cosmocil CQ[â] is a polyaminopropyl biguanide-based antimicrobial agent that has a broad spectrum of activity, acting on gram-positive and gram-negative micro-organisms such as *pseudonomas, S. aureus, E. coli*, yeasts, fungi, and viruses without causing selection of resistant mutants. It is a formaldehyde-free, water soluble, chemically stable and nonvolatile solution that can be stored for long periods. It has low cell toxicity when used on skin or in the oral cavity, even for long periods, and it is not genotoxic, carcinogenic, or teratogenic. Like all biguanides, Cosmocil CQ[â] acts on the microbial cytoplasmatic membrane, altering cell permeability and causing precipitation of its content. Cosmocil CQ[â] has been used as an antimicrobial agent in several skin care creams and lotions, hair conditioner and shampoos, deodorants, moist cleaning tissues, dressings, cosmetics, and contact lens cleaning products, among others.[31,32]

Myacide pharma BP[â] (bronopol) is a biocide previously associated with Cosmocil CQ[â] to increase the antimicrobial potential of the agent. The experimental solution also has polyvinylpirrolidone K30 in its formulation. This component acts as a disperser and suspensor, and has fixation and pellicle formation properties,[33] which helps improve fixation of the solution on toothbrush bristles and increase the contact time of the antimicrobial agent.

The association of these basic ingredients resulted in the experimental solution evaluated in this study. Although the experimental solution did not present the largest inhibition halos in the agar diffusion test performed for the randomized clinical trial, its efficacy against the formation of MS colonies/biofilms was statistically similar to that of Periogard (100%; $P>.05$). In spite of these promising results, however, further research should be conducted to reach an ideal formulation that can completely eliminate microorganisms from toothbrushes.

Brushtox antiseptic cleanser was developed in England in 1998 specifically for disinfection of toothbrush bristles. According to Carter and Paterson,[34] this agent is highly effective against bacteria, fungi, and viruses. This study's findings showed that Brushtox had 73% efficacy against the formation of MS colonies/biofilms on the surface of the bristles and was less effective than Periogard and the

experimental solution ($P<.01$).

In a recent in vitro investigation, Neal and Rippin[27] evaluated the antimicrobial effect of Brushtox by the agar diffusion test and found an inhibition halo average of 22.9 mm. In the present study, an inhibition halo average of 7.8±6.3 mm was observed for Brushtox. This difference may possibly be attributed to the following reasons:

1. Neal and Rippin[27] used 10-mm diameter wells, while 5-mm diameter wells were used in this study.
2. The antimicrobial activity of Brushtox against some microorganisms was null in this study, which reduced the average of inhibition halos observed with the use of this solution.

The outcomes of this investigation highlight that, due to the contamination of toothbrushes by a wide array of microorganisms and considering the efficacy of antimicrobial sprays for preventing microbial accumulation and growth, the need for disinfection of toothbrushes after every brushing should be widely spread and strongly emphasized.

CONCLUSIONS

After a single brushing performed by a dentist on children 24 to 48 months old with mutans streptococci (MS) detected in their saliva, severe contamination by MS colonies/biofilms was observed on the bristles of all (100%) toothbrushes sprayed with sterile tap water (control). Although Brushtox presented better results than the sterile tap water, Periogard and the experimental solution showed greater efficacy against the formation of MS colonies/biofilms on the toothbrush bristles and exhibited larger microbial growth inhibition halos.

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