Molecular detection of in-vivo microbial contamination of metallic orthodontic brackets by checkerboard DNA-DNA hybridization

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Introduction: Knowing the microbiota that colonizes orthodontic appliances is important for planning strategies and implementing specific preventive measures during treatment. The purpose of this clinical trial was to evaluate in vivo the contamination of metallic orthodontic brackets with 40 DNA probes for different bacterial species by using the checkerboard DNA-DNA hybridization (CDDH) technique. **Methods:** Eighteen patients, 11 to 29 years of age having fixed orthodontic treatment, were enrolled in the study. Each subject had 2 new metallic brackets bonded to different premolars in a randomized manner. After 30 days, the brackets were removed and processed for analysis by CDDH. Data on bacterial contamination were analyzed descriptively and with the Kruskal-Wallis and Dunn post tests ($\alpha = 0.05$). Forty microbial species (cariogenic microorganisms, bacteria of the purple, yellow, green, orange complexes, "red complex + *Treponema socranskii*," and the cluster of Actinomyces) were assessed. **Results:** Most bacterial species were present in all subjects, except for *Streptococcus constellatus*, *Campylobacter rectus*, *Tannerella forsythia*, *T socranskii*, and *Lactobacillus acidophillus* (94.4%), *Propionibacterium acnes I* and *Eubacterium nodatum* (88.9%), and *Treponema denticola* (77.8%). Among the cariogenic microorganisms, *Streptococcus mutans* and *Streptococcus sobrinus* were found in larger numbers than *L acidophillus* and *Lactobacillus casei* ($P < 0.001$). The periodontal pathogens of the orange complex were detected in larger numbers than those of the "red complex + *T socranskii*" ($P < 0.0001$). Among the bacteria not associated with specific pathologies, *Veillonella parvula* (purple complex) was the most frequently detected strain ($P < 0.0001$). The numbers of yellow and green complex bacteria and the cluster of Actinomyces were similar ($P > 0.05$). **Conclusions:** Metallic brackets in use for 1 month were multi-colonized by several bacterial species, including cariogenic microorganisms and periodontal pathogens, reinforcing the need for meticulous oral hygiene and additional preventive measures to maintain oral health in orthodontic patients. (Am J Orthod Dentofacial Orthop 2012;141:24-9)

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Fixed orthodontic treatment promotes specific alterations in the oral enviroment,[1] including decreased pH, increased plaque accumulation,[2] and elevation of microbial counts in the saliva and the biofilm.[3,4] Higher levels of oral microorganisms increase not only the risk of caries and periodontal diseases,[1,5] but also the chances of systemic complications,[6-9] since certain orthodontic procedures can cause transient bacteremias.[10-12]

Although bacterial contamination on components of fixed and removable orthodontic appliances has been investigated, most studies have used microbial culture techniques[13-17] and assessed cariogenic microorganisms.[18-22]

The advent of molecular biology techniques represented an important advance in microbiology research and made possible more precise identification of bacterial species by using DNA probes. Molecular genetic methods, such as polymerase chain reaction[20] and

checkerboard DNA-DNA hybridization (CDDH),[23] do not rely on retrieval of specimens under carefully controlled anaerobic conditions, nor do they require special transportation media or cultivation of isolates. CDDH was introduced as a method for hybridizing large numbers of DNA samples against large numbers of DNA probes on a single support membrane. It is faster than polymerase chain reaction because it uses several DNA probes at the same time and allows for simultaneous determination of the presence of many bacterial species in single or multiple clinical samples, which can be stored for long periods of time.[23] CDDH has been used in different areas of dental research such as periodontology,[24] endodontics,[25] implantology,[26] pediatric dentistry,[27] and cariology.[28] In orthodontics, it has only been used in 1 study that evaluated microbial contamination in metallic and ceramic brackets.[1]

Knowing the microbiota that colonizes orthodontic appliances is important for planning strategies and implementing specific preventive measures for control during orthodontic treatment. Therefore, in this clinical trial, we evaluated the in-vivo contamination of metallic orthodontic brackets with 40 DNA probes for different bacterial species, using the CDDH technique.

MATERIAL AND METHODS

Eligible participants were selected from patients of both sexes with complete permanent dentitions; they were nonsmokers without dental caries or periodontal disease who were under orthodontic treatment with fixed appliances for less than 16 months, had good general health, and had not used antibiotics or antimicrobial mouthwashes within 3 months before the study. Eighteen patients (ages, 11-29 years; mean, 15.5 years; 11 male, 7 female) who met these inclusion criteria were enrolled as participants. The study purposes were fully explained to the patients or their legal representatives, who signed an informed consent form for participation. The research protocol was reviewed and approved by the research ethics committee of the School of Dentistry of Ribeirão Preto, University of São Paulo (process number 2008.1.163.58.8).

One week before the beginning of the study, each patient's plaque index was determined by 1 operator (M.C.D.A.) according to the method of Silness and Löe[29] to limit the range of plaque levels of the patients at the beginning of the study. Subjects within a range of initial average dental plaque from 0.5 to 1.5 were included (mean plaque level, 0.91; SD, 0.30). Next, plaque deposits were eliminated with meticulous rubber cup and pumice prophylaxis. The patients were instructed to brush their teeth 3 times a day after meals using a toothbrush (Professional, Colgate-Palmolive Indústria and Comércio, São Paulo, São Paulo, Brazil) and a fluoride-containing dentifrice (Colgate Máxima Proteção Anticariess, Colgate-Palmolive Indústria and Comércio) supplied by the researchers.

In all patients, 2 new, sterile edgewise metallic orthodontic brackets (0.022 × 0.028-in slot) (Generus, GAC International, Bohemia, NY) were bonded with orthodontic light-cured adhesive (Transbond XT, 3M Unitek, Monrovia, Calif) to premolars (maxillary right and left, or mandibular right and left) selected randomly by using the Statistical Analysis Systems (version 9.1.3 for Windows; SAS Institute, Cary, NC) software.

After 30 days, the brackets were removed by an orthodontist (M.C.D.A.) in a blinded fashion. Each bracket was placed into a labeled plastic tube containing 150 μL of Tris EDTA (TE) buffer solution (pH 7.6) and 100 μL of 0.5M sodium hydroxide (NaOH), and agitated for 30 seconds (Mixtron; Toptronix, São Paulo, São Paulo, Brazil) for microbial detachment. The brackets were collected with sterile clinical pliers, and the plastic tubes containing the bacterial suspension were stored frozen at −20°C for further analysis by CDDH.

The presence and total counts of 40 bacterial species in the brackets were determined by CDDH.[23,30,31] Genomic DNA probes for bacteria belonging to the microbial complexes described by Haffajee et al[30] (purple, yellow, green, orange, and red + *Treponema socranskii* complexes and the cluster of Actinomyces) and cariogenic bacteria were used. Haffajee et al examined the microbial complex communities in the supragingival plaque and observed that *T socranskii* was somehow associated with the periodontal pathogens of the red complex. For this reason, the designation "red complex + *T socranskii*" will be used throughout this article for the purposes of this study.

The collected samples were boiled for 10 minutes to cause cell lysis and denaturation, and neutralized with 0.8 mL of 5 mol/L of ammonium acetate. The released DNA was then fixed in individual lanes of a positively charged nylon membrane (Boehringer Mannheim, Indianapolis, Ind) by using a checkerboard slot blot device (Minislot 30, Immunetics, Cambridge, Mass). Forty digoxigenin-labeled whole genomic DNA probes (Roche Applied Science, Indianapolis, Ind) were constructed and hybridized perpendicularly to the lanes of the clinical samples by using a Miniblotter 45 apparatus (Immunetics). Bound probes were detected by using phosphatase-conjugated antibody to digoxigenin (Roche Applied Science). After incubation in a solution containing the CDP-Star substratum (Amersham Pharmacia Biotech, Buckinghamshire, England), the membranes were placed in an autoradiography cassette under a radiographic film (X-Omat; Kodak, Rochester,

NY), which was developed for chemiluminescence signal detection. Signals were evaluated visually by comparing to the standards of 10^5 and 10^6 bacterial cells of the test species on the last 2 lanes of the same membrane. This provided the approximate number of bacterial cells per sample for each bacterial strain evaluated; this was equal to the sum of the values obtained in the 2 brackets removed from each patient. The data were read twice by a blinded examiner (M.F.) (kappa, >0.8). The sensitivity of this assay was settled to allow detection of 10^4 cells of a bacterial species by adjusting the concentration of each DNA probe. This procedure was carried out to provide the same sensitivity of detection for all species (ie, the concentrations were adjusted so that all probes had a similar signal intensity). To facilitate the semiquantitative examination of chemiluminescence signals for each microorganism in each sample, the intensity of the contamination of the brackets by the different bacterial species was evaluated at the following levels: 0 (not detected), 1×10^4, 1×10^5, 5×10^5, 1×10^6, and 1×10^7.

Statistical analysis

The results obtained with the CDDH technique were analyzed descriptively by using the SAS statistical software to evaluate the level of contamination of the brackets by each of the 40 tested microorganisms. Prevalence of each microorganism was calculated as well as the general composition of the microbiota on the metallic brackets, considering the total numbers of microorganisms found in all subjects. Medians and quartiles were used to describe bacteria distribution, since most of them were not normally distributed. Comparison among bacterial distribution and also complexes were done by using the Kruskal Wallis test and the Dunn post-hoc test, with the Graphpad Prism for Windows (version 5.0; Graphpad Software, San Diego, Calif) statistical software. A significance level of 5% was set for all analyses.

RESULTS

Most bacterial species evaluated were present in all subjects, except for *Streptococcus constellatus*, *Campylobacter rectus*, *Tannerella forsythia*, *T socranskii*, and *Lactobacillus acidophilus*, which were present in 94.4% of the patients; *Propionibacterium acnes I* and *Eubacterium nodatum*, which were detected in 88.9%; and *Treponema denticola*, the least prevalent of the bacterial species, found in 77.8% of the patients.

The general composition of the microbiota on metallic brackets is graphically illustrated in the Figure. The total counts of the 40 bacterial species in the brackets ranged from 3.425×10^7 to 1.8813×10^8 (median, 7.506×10^7). The distribution of the total numbers of microorganisms of the 40 bacterial species on the orthodontic brackets is presented in the Table.

When we analyzed each microbial complex separately, the periodontal pathogens of the orange complex were present in larger numbers than those of the "red complex + *T socranskii*" (P <0.0001). Among the other microorganisms not associated with specific pathologies, *Veillonella parvula* (purple complex) was present in the largest numbers (P <0.0001). No statistically significant differences (P >0.05) were found among the yellow complex, green complex bacteria, and the cluster of Actinomyces.

Among the cariogenic bacteria, *Streptococcus mutans* and *Streptococcus sobrinus* were found in larger numbers than *L acidophillus* and *Lactobacillus casei* (P <0.001).

Regarding the cluster of Actinomyces, *Actinomyces naeslundii II* had the greatest counts (P <0.001). Among the bacteria of the yellow complex, *Streptococcus gordoni*, *Streptococcus sanguinis*, *Streptococcus mitis*, *Streptococcus oralis*, and *Leptotrichia buccalis* were present in the largest numbers (P <0.05). Regarding the 3 bacterial species of the green complex, *Capnocytophaga gingivalis* was found in larger numbers than the others, followed by *Eikenella corrodens* and *Capnocytophaga sputigena*. However, no statistically significant difference (P >0.05) was found between *C gingivalis* and *C sputigena*. All orange complex bacteria were found in high counts in the subjects of this study, without significant differences among them (P >0.05), except for *Prevotella intermedia*, *Campylobacter gracilis*, and *C rectus*. Regarding the red complex bacteria, *T forsythia* and *Porphyromonas gingivalis* (P <0.05) were present in the largest numbers. *T socranskii* counts were also high (P <0.05).

DISCUSSION

The CDDH technique used in this study allows for evaluating a larger number of microbial species in the same experiment, thus making it possible to establish a profile of the microbial contamination in metallic brackets during orthodontic treatment.

The bacterial species evaluated in this study are frequently found in the mouth, sometimes specifically associated with bacteremias and pathologies during orthodontic treatment, such as dental caries and periodontal disease. To facilitate data collection and interpretation, the bacterial species analyzed, except for the cariogenic microorganisms, were divided according to the microbial complexes in the supragingival biofilm,

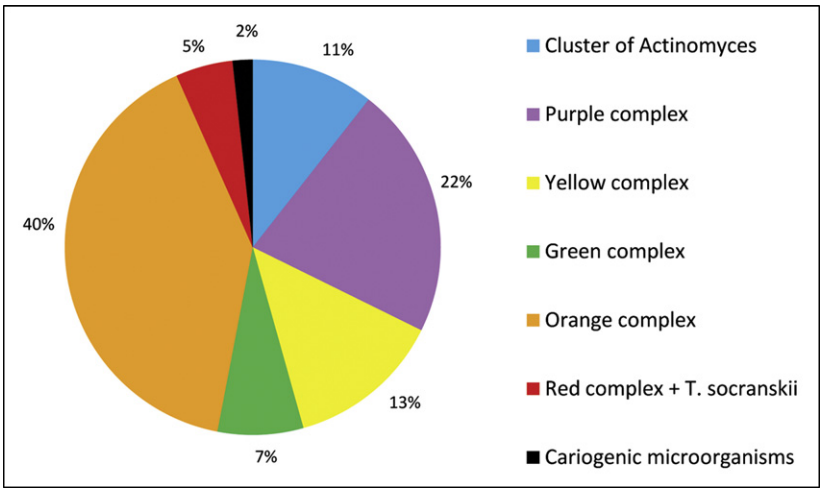

Fig. General composition of microbiota on metallic brackets.

as defined by Haffajee et al[30] (bacteria of the purple, yellow, green, orange complexes, "red complex + *T socranskii*," and the cluster of Actinomyces), considering that the brackets are bonded to the buccal surfaces of the teeth, near the gingival margin.

The literature is scarce in studies evaluating the bacterial contamination in specific components of orthodontic appliances using molecular techniques, such as polymerase chain reaction[20] and CDDH[1]; this limits comparisons of our results with the literature.

Anhoury et al[1] used CDDH to compare the total bacterial counts on metallic and ceramic orthodontic brackets bonded to maxillary incisors and premolars, which were collected from orthodontic patients at debonding after nonreported treatment periods. According to the results, the type of bracket did not influence on the bacterial counts. The cariogenic microorganisms evaluated by those authors, *S mutans* and *L acidophillus*, were present in similar numbers. In our study, *S mutans* and *S sobrinus* were found in larger numbers than *L acidophillus* and *L casei* ($P < 0.001$). Ahn et al[20] recently used polymerase chain reaction to evaluate the prevalence of mutans streptococci adhering to maxillary and mandibular metallic incisor brackets collected from patients at debonding. The prevalence of *S mutans* was about 2 times higher than that of *S sobrinus*. In our study, CDDH did not show significant differences between the counts of these microorganisms.

The bacterial species of the yellow, green, and purple complexes and the cluster of Actinomyces are not associated with specific diseases, and usually colonize the plaque before the orange and red complex bacteria.[31] According to Anhoury et al,[1] bacteria of the cluster of Actinomyces were found in larger numbers than those of the yellow, green, and purple complexes. Our findings were somewhat different. *V parvula*, a microorganism of the purple complex, was found in larger numbers than those of the other complexes ($P < 0.0001$), and the bacteria of the cluster of Actinomyces were present in similar numbers as the green and yellow complex bacteria ($P > 0.05$).

Bacterial species of the red and orange complexes are involved in the development of periodontal disease[30,31] and are directly associated with the gingival inflammation commonly observed during orthodontic treatment.[32,33] In this study, the orange complex species were the most prevalent bacteria on the metallic brackets, representing 40% of the total bacterial counts, reinforcing their involvement in gingival inflammation, considering their proximity to the gingiva.

On the other hand, there were lower counts of red complex species compared with orange complex species ($P < 0.0001$). Similar results were found by Anhoury et al,[1] except for *E nodatum*. According to Socransky et al,[31] colonization by orange complex bacteria precedes colonization by red complex bacteria; this could explain the greater counts of orange complex species in this study. In addition, the differences observed between our results and those of Anhoury et al[1] might be explained by the fact that those authors evaluated brackets debonded at the end of the orthodontic treatment—ie, after long periods in the mouth; this might have contributed to increasing the colonization by certain microorganisms.

Considering the wide array of bacterial species found on metallic brackets in vivo, further studies are needed to guide the establishment of preventive clinical protocols that can be effective in controlling microbial

Table. Distribution of the total numbersof microorganisms of the 40 bacterial species on the orthodontic brackets after 30 days of clinical use

Microorganism	*M (Q1-Q3)*	*Microorganism*	*M (Q1-Q3)*
Cariogenic microorganisms		Green complex	
L acidophilus (*ATTC 4356*)	1.0×10^5 (2.0×10^4-1.1×10^5)	*C gingivalis* (*ATTC 33624*)	1.5×10^6 (1.0×10^6-2.0×10^6)
L casei (*ATTC 393*)	2.0×10^4 (2.0×10^4-2.0×10^4)	*C sputigena* (*ATTC 33612*)	6.0×10^5 (2.0×10^5-1.0×10^6)
S mutans (*ATTC 25175*)	8.0×10^5 (2.0×10^5-1.0×10^6)	*E corrodens* (*ATTC 23834*)	1.0×10^6 (6.0×10^5-1.5×10^6)
S sobrinus (*ATTC 33748*)	6.0×10^5 (2.0×10^5-1.0×10^6)	Orange complex	
Cluster of Actinomyces		*C gracilis* (*ATTC 33236*)	1.0×10^6 (6.0×10^5-1.0×10^6)
A gerencseriae (*ATTC 23860*)	1.0×10^6 (6.0×10^5-1.0×10^6)	*C rectus* (*ATTC 33238*)	6.0×10^5 (2.0×10^5-1.0×10^6)
A israelii (*ATTC 12102)*	6.0×10^5 (6.0×10^5-1.0×10^6)	*C showae* (*ATTC 51146*)	1.0×10^6 (6.0×10^5-1.5×10^6)
A naeslundii I (*ATTC 12104*)	6.0×10^5 (6.0×10^5-1.5×10^6)	*C ochracea* (*ATTC 33596*)	1.0×10^6 (6.0×10^5-2.0×10^6)
A naeslundii II (*ATTC 43146*)	1.75×10^6 (1.0×10^6-1.1×10^7)	*F nucleatum sp nucleatum* (*ATTC 25586*)	1.5×10^6 (1.1×10^6-1.1×10^7)
A odontolyticus I (*ATTC 17929*)	6.0×10^5 (2.0×10^5-1.0×10^6)	*F nucleatum sp vincentii* (*ATTC 49256*)	1.25×10^6 (1.0×10^6-1.5×10^6)
Purple complex		*F nucleatum sp polymorphum* (*ATTC 10953*)	1.5×10^6 (1.0×10^6-1.5×10^6)
V parvula (*ATTC 10790*)	2.0×10^7 (2.0×10^7-2.0×10^7)	*F periodonticum* (*ATTC 33693*)	1.5×10^6 (1.5×10^6-1.1×10^7)
Yellow complex		*P intermedia* (*ATTC 25611*)	1.0×10^6 (5.0×10^5-1.0×10^6)
A actinomycetemcomitans [*ATTC 43718(a), 29523(b)*]	6.0×10^5 (2.0×10^5-1.0×10^6)	*P melaninogenica* (*ATTC 25845*)	2.0×10^6 (1.5×10^6-2.0×10^7)
L buccalis (*ATTC 14201*)	1.0×10^6 (6.0×10^5-1.0×10^6)	*P nigrescens* (*ATTC 33563*)	1.0×10^6 (1.0×10^6-1.5×10^6)
P acnes [*ATTC 11827(a), 11828(b)*]	6.0×10^4 (2.0×10^4-2.0×10^5)	*S noxia* (*ATTC 43541*)	1.0×10^6 (6.0×10^5-1.5×10^6)
S anginosus (*ATTC 33397)*	6.0×10^5 (2.0×10^5-6.0×10^5)	"Red complex + *T socranskii*"	
S constellatus (*ATTC 27823*)	1.1×10^5 (2.0×10^4-6.0×10^5)	*E nodatum* (*ATTC 33099*)	2.0×10^4 (1.0×10^4-1.1×10^5)
S gordonii (*ATTC 10558*)	1.5×10^6 (1.0×10^6-2.0×10^6)	*P gingivalis* (*ATTC 33277*)	2.0×10^5 (1.1×10^5-2.0×10^5)
S intermedius (*ATTC 27335*)	6.0×10^5 (2.0×10^5-1.0×10^6)	*T forsythia* (*ATTC 43037*)	2.0×10^5 (2.0×10^5-1.0×10^6)
S mitis (*ATTC 49456*)	1.0×10^6 (1.0×10^6-1.5×10^6)	*T denticola* (*B1*)	1.0×10^5 (1.0×10^4-2.0×10^5)
S oralis (*ATTC 35037*)	1.0×10^6 (6.0×10^5-1.0×10^6)	*T socranskii* (*S1*)	6.0×10^5 (2.0×10^5-1.5×10^6)
S sanguinis (*ATTC 10556*)	1.5×10^6 (1.0×10^6-1.5×10^6)		

ATTC, American Type Culture Collection; *M*, median; *Q1*, first quartile; *Q3*, third quartile.

contamination and prevent the development of bacteremias and pathologies, such as dental caries and periodontal disease, during orthodontic treatment.

CONCLUSIONS

Analysis by the molecular technique showed that metallic brackets were multi-colonized by several bacterial species (cariogenic microorganisms, bacteria belonging to the purple, yellow, green, orange complexes, "red complex + *T socranskii*," and the cluster of Actinomyces) after a short period of placement (1 month), reinforcing the need for meticulous oral hygiene and additional preventive measures to maintain oral health in orthodontic patients.

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