Quantitative analysis of changes in salivary mutans streptococci after orthodontic treatment

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Introduction: The purpose of this study was to analyze the initial changes in salivary mutans streptococci levels after orthodontic treatment with fixed appliances. **Methods:** Our subjects consisted of 58 adults. Whole saliva and simplified oral hygiene index values were obtained at 4 time points: at debonding (T1), 1 week after debonding (T2), 5 weeks after debonding (T3), and 13 weeks after debonding (T4). Repeated measures analysis of variance was used to determine the time-related differences in salivary bacterial levels and the simplified oral hygiene index values among the 4 time points after quantifying the salivary levels of *Streptococcus mutans*, *Streptococcus sobrinus*, and total bacteria with real-time polymerase chain reaction. **Results:** Simplified oral hygiene index values and total bacteria significantly decreased, but salivary mutans streptococci levels significantly increased after orthodontic treatment. The amounts of total bacteria in saliva significantly decreased at T3 (T1, T2 > T3, T4), and the simplified oral hygiene index values decreased at T2 (T1 > T2, T3, T4). However, salivary *S mutans* and *S sobrinus* significantly increased at T3 and T4, respectively (T1, T2 < T3 < T4). Furthermore, the proportion of mutans streptococci to total bacteria significantly increased at T4 (T1, T2, T3 < T4). **Conclusions:** This study suggests that careful hygienic procedures are needed to reduce the risk for dental caries after orthodontic treatment, despite overall improved oral hygiene status. (Am J Orthod Dentofacial Orthop 2014;145:603-9)

One of the most common side effects during orthodontic treatment is enamel demineralization around fixed appliances.[1] Mutans streptococci (MS) are generally considered the major cause of enamel demineralization because of their ability to adhere to tooth surfaces and produce lactic acid through carbohydrate fermentation.[2-4] Among them, *Streptococcus mutans* and *Streptococcus sobrinus* are commonly found in the oral cavity.[5,6] Therefore, it is important to understand the changes in *S mutans* and *S sobrinus* numbers to prevent enamel demineralization in orthodontic patients.

Various methods have been used to identify MS, including colony morphology on mitis-salivarius bacitracin agar, biochemical tests, and immunologic methods. However, these techniques are inaccurate, time-consuming, or laborious. Recently, real-time polymerase chain reaction (PCR) has emerged as a more rapid and sensitive method of quantifying and detecting specific bacterial species. Real-time PCR can detect absolute numbers of targeted bacteria, and the various applications including amplification, measurement, and quantification can be conducted simultaneously to minimize the chances of contamination.[7]

Patients with fixed orthodontic appliances are subject to changes in the oral environment, including increased MS and decreased pH, because MS preferentially colonize teeth and orthodontic appliances, and the complex designs of orthodontic appliances impede proper access for cleaning.[8-11] Although the changes in MS during orthodontic treatment have been extensively investigated, few studies have investigated changes in MS after orthodontic treatment. Considering the importance of MS levels in determining the risk of enamel demineralization and

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dental caries, we undertook this study to investigate changes in MS levels after orthodontic treatment to help determine caries risk during the retention period. The purpose of this in-vivo prospective study was to analyze the changes in salivary levels of *S mutans*, *S sobrinus*, and total bacteria after orthodontic treatment with fixed appliances using real-time PCR.

MATERIAL AND METHODS

This study initially comprised 63 subjects who were poised to finish orthodontic treatment with fixed appliances in Department of Orthodontics of Seoul National University Dental Hospital in Korea. Unfortunately, 5 subjects did not attend their periodic appointments. The study population finally consisted of 58 patients (20 men, 38 women; mean age, 23.4 years). The inclusion criteria at the start of this experiment were (1) adults older than 19 and 17 years for men and women, respectively, (2) permanent dentition of more than 24 teeth, (3) longer than a 12-month treatment period (average, 19.6 months), and (4) treated with 1 of the following bracket types with a 0.022-in slot: Clarity SL (3M Unitek, Monrovia, Calif; n = 22), Clippy-C (Tomy, Tokyo, Japan; n = 24), and Damon Q (Ormco, Orange, Calif; n = 12). The reason for recruiting subjects with 3 bracket types was to acquire a sufficient sample size. The exclusion criteria were (1) any systemic disease, (2) any active carious lesions, (3) poor oral hygiene (simplified oral hygiene index value >3.0),[12] and (4) topical fluoride application (except for fluoridated dentifrice) or antibacterial therapy within the last 6 months.

Immediately before debonding of the orthodontic appliances, 0.0175-in twist-wire fixed lingual retainers were attached to the anterior segments of both arches in most subjects (43 of 58). All patients received maxillary and mandibular removable retainers after debonding and were asked to wear their retainers 24 hours a day. The subjects received oral hygiene instructions, including brushing and flossing, and maintenance methods for the removable retainers with mechanical brushing with a toothbrush and rinsing with running tap water. All subjects signed informed consent forms, and the institutional review board of the Seoul National University Hospital approved the study protocol.

Unstimulated whole saliva was collected by the spitting method as previously described.[13] All subjects were asked to refrain from eating, drinking, toothbrushing, or mouth rinsing at least 2 hours before saliva collection. Unstimulated whole saliva was collected at the following 4 time points according to common retention protocols as previously reported[14]: at debonding (T1), 1 week after debonding when the patients began to wear the removable retainers (T2), 5 weeks after debonding (T3), and 13 weeks after debonding (T4). The simplified oral hygiene index values, which measure oral hygiene status using debris and calculus deposition from 2 anterior and 4 posterior teeth, were examined by 1 investigator (W.-S.J.) at each time point.[12]

One milliliter of unstimulated whole saliva was centrifuged at 13,000 rpm for 10 minutes. After removing the supernatant, the pellet was washed 3 times with 1.0 mL of cold phosphate-buffered saline solution (pH = 7.4). The pellet was resuspended with 1.0 mL of cold phosphate-buffered saline solution and homogenized by sonication with three 30-second pulses with 30-second intermittent cooling stages in a chilled ice box.

Bacterial chromosomal DNA was extracted using a CellEase Bacteria II Genomic DNA Extraction Kit (Biocosm, Osaka, Japan) according to the manufacturer's instructions after mutanolysin (Sigma-Aldrich, St Louis, Mo) treatment for 30 minutes at 37°C. A NanoVue spectrophotometer (General Electric Healthcare Life Sciences, Pittsburgh, Pa) was used to assess the quality of the extracted DNA after preparation.

Known specific primers that amplify the dextranase genes of *S mutans* and *S sobrinus* were designed from the *gtfB* and *gtfU* genes, respectively (Table I).[15,16] A conserved sequence in the 16S rRNA was selected to quantify the numbers of total bacteria.[17] All primers were commercially synthesized (Takara-Korea, Seoul, Korea). To test primer specificities, 11 strains of MS and other gram-positive and gram-negative species were examined using the specific primer pairs (Table II).

DNA was extracted from *S mutans* UA159 and *S sobrinus* SL1 to generate standard curves. DNA concentration was estimated by absorbance at 260 nm, and series of 10-fold dilutions from 10^3 to 10^9 copies were prepared for standard curves as previously described.[18] The amount of bacterial DNA in the samples was extrapolated from the standard curve.

Real-time PCR was performed using the iQ5 system (Bio-Rad, Hercules, Calif). The reaction mixtures contained 2 μL of purified DNA from the saliva samples, 100 pmol of primer, and 10 μL of 2x iQ SYBR Green Supermix (Bio-Rad). Distilled water was added to a final volume of 20 μL. The samples were subjected to an initial amplification for 30 seconds at 94°C, 40 cycles of denaturation for 20 seconds at 95°C, primer annealing for 45 seconds at 60°C, and extension for 10 seconds at 60°C. All data, including the amounts of total bacteria, *S mutans*, and *S sobrinus*, were analyzed with iQ5 Optical System Software (Bio-Rad). All experiments for quantifying the bacteria were performed in triplicate and independently repeated twice.

Repeated measures analysis of variance (ANOVA) was used to determine the time-related differences in the