

Changes in Subgingival Microflora after Placement and Removal of Fixed Orthodontic Appliances

Marija Živković Sandić¹, Branka Popović², Jelena Čarkić², Nadja Nikolić², Branislav Glišić¹

¹Clinic for Orthodontics, School of Dental Medicine, University of Belgrade, Belgrade, Serbia

²Institute of Human Genetics, School of Dental Medicine, University of Belgrade, Belgrade, Serbia

SUMMARY

Introduction The placement of fixed orthodontic appliances may lead to increased plaque accumulation and changes in subgingival microflora.

Objective The aim of this study was to examine the changes in frequency of subgingival microflora that occur after placement and removal of fixed orthodontic appliance using polymerase chain reaction (PCR).

Methods This study included 33 orthodontic patients, who were divided into two groups. Subgingival plaque samples were collected from the right upper incisor (U1) and right upper first molar (U6). In group A, the samples were taken three times: before placement appliance (T1), after one month (T2), and after 3 months (T3). In group B the samples were also taken three times: before appliance removal (T1), after one month (T2), and after three months (T3). PCR method was used to determine the presence of *P. gingivalis*, *A. actinomycetemcomitans*, *T. forsythia*, and *P. intermedia*.

Results In group A the frequency of *P. gingivalis* showed statistically significant decrease at U1 ($p=0.049$) and U6 ($p=0.008$), from T1 to T2, and at U1 ($p=0.048$) from T1 to T3. In group B only the frequency of *T. forsythia* showed a statistically significant decrease, at U6 (T1 vs. T2, $p=0.004$; T1 vs. T3, $p=0.0003$). Regarding other analyzed bacteria, changes in the presence were noticed but no statistical significance was found.

Conclusion Placement of fixed appliances may have an impact on subgingival microflora, but in the first months after the placement and removal of the appliance changes were not significant, probably due to good oral hygiene.

Keywords: orthodontic appliance; bacteria; PCR

INTRODUCTION

Fixed orthodontic appliance is the most common method for treating malocclusions in contemporary orthodontics. However, the placement of orthodontic brackets and bands may compromise oral hygiene, because new retentive places are formed resulting in increased accumulation of dental plaque leading to gingival inflammation [1, 2]. As known, bacterial plaque is the main etiological factor for the development of gingival inflammation and periodontitis [3, 4].

Numerous studies have registered changes of microbiologic status during orthodontic therapy and after the removal of orthodontic fixed appliance [2, 5-8]. However, some studies have reported that the placement of fixed orthodontic appliances affects subgingival microflora by increasing the prevalence of periodontopathogens [5, 6]. Also, some other studies have reported that anaerobic bacteria are significantly reduced after appliance removal [9, 10]. Contrary to those data, the results of ten-year retrospective studies have shown that orthodontic treatment during adolescence has no significant effect on later periodontal health [7].

Moreover, one recent prospective study found that placement of fixed appliances has an impact on periodontal parameters, but these changes are partially reversible two years after the end of treatment [11].

OBJECTIVE

The aim of this study was to examine the changes that occur in the subgingival microflora after placement and removal of fixed orthodontic appliance using PCR.

METHODS

Subjects and clinical procedures

This study was carried out on 33 patients (14 males and 19 females), aged between 12 and 36 years. The mean age of all patients was 19.7 years (females 19.2 and males 20.5). They were enrolled according to the following criteria: fixed orthodontic appliance in the upper tooth arch and healthy systemic condition. The subjects were divided into 2 groups. Group A consisted of patients at the beginning and group B of the patients at the end of orthodontic therapy.

In group A the samples were taken at three different times: before placement of fixed appliance (T1), one month after the placement (T2), and three months after the placement (T3). In group B the samples were also taken at three times: before appliance removal (T1), one month after appliance removal (T2), and three months after appliance removal (T3). After the placement of appliance, the patients from group A were instructed to take care of

Correspondence to:

Branka POPOVIĆ
Institute of Human Genetics
School of Dental Medicine
Dr Subotića 8, 11000 Belgrade
Serbia
brankapopovic7@yahoo.com

oral hygiene by brushing their teeth more often and longer, but without additional use of antibacterial mouthwashes. Also, patients from group B, after removal of appliance were advised to visit their dentist to have plaque and calculus removed and their teeth polished.

Subgingival plaque samples were collected from subgingival space at the buccal, mesial and distal side of the right upper incisor (U1) and the right upper first molar (U6). The sampling sites were fixed with cotton rolls and dried by gentle air stream. Sterile paper points were placed into the subgingival space depth of about 1mm and left in situ for 15 seconds. Paper points were transferred into a sterile tube and then kept in freezer at -20°C.

Bacteriological methods

The extraction of potentially present bacterial DNA was performed by the boiling method of Gebara et al. [12]. The presence of the most important periodontopathogens: *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia* and *Prevotella intermedia*, was determined using the PCR method. The sequence of primers for 16S rRNA gene of analyzed bacteria is shown in Table 1.

PCR mixture in a total volume of 25 µl contained 1 µl of 5 µM up-stream and down-stream primers, 5 µl of 10×PCR buffer, 1 µl of 0.2 mM dNTP mix, 1 unit of Taq polymerase (Fermentas, Vilnius, Lithuania), 3 µl of bacterial DNA, and distilled water up to 25 µl. The number of amplification cycles was 35 performed in a thermal cycler

Table 1. PCR primers

Primers	Size (bp)
<i>Universal 16S rDNA</i> 5' AGA GTT TGA TCC TGG CTC AG 3'	
<i>Porphyromonas gingivalis</i> 5' CAA TAC TCG TAT CGC CCG TTA TTC 3'	400
<i>Aggregatibacter actinomycetemcomitans</i> 5' CAC TTA AAG GTC CGC CTA CGT GC 3'	600
<i>Tannerella forsythia</i> 5' GTA GAG CTT ACA CTA TAT CGC AAA CTC CTA 3'	840
<i>Prevotella intermedia</i> 5' GTT GCG TGC ACT CAA GTC CGC C 3'	660

(PCR Express, Hybaid, USA). The temperature conditions of amplification consisted of initial denaturation of 3 minutes at 94°C. Each of the 35 amplification cycles consisted of denaturation at 94°C for 1 min, hybridization for 1 min at 55°C, and extension for 3 min at 72°C. The final extension lasted for 7 minutes at 72°C.

PCR products were run on an 8% polyacrylamide gel, and after electrophoresis, the gel was stained with ethidium bromide solution. Bacterial DNA was observed after staining under transilluminator UV light (Power Station 300 plus, Labnet International, Inc.).

Statistical analysis

Comparison between the groups was performed using a two-tails Chi-square (χ^2) test with Yates' correction and Student's t-test. The significance was set at p value of <0.05.

Table 2. Frequency of periodontopathogens in subgingival plaque at three times: before placement of fixed appliance (T1), one month after the placement (T2), and 3 months after the placement (T3)

Group A		T1		T2		T3		p-value		
Microorganism	Tooth	N	%	N	%	N	%	T1 vs. T2	T2 vs. T3	T1 vs. T3
<i>Aggregatibacter actinomycetemcomitans</i>	U1	14	50.0	7	50.0	8	57.1	0.65	0.49	0.49
	U6	14	85.7	11	78.6	12	85.7	0.49	0.50	0.70
<i>Porphyromonas gingivalis</i>	U1	14	28.6	0	0	0	0	0.049*	/	0.048*
	U6	14	42.8	0	0	2	14.3	0.008*	0.24	0.10
<i>Prevotella intermedia</i>	U1	14	42.8	9	64.3	7	50.0	0.23	0.35	0.49
	U6	14	57.1	9	64.3	6	42.8	0.49	0.23	0.35
<i>Tannerella forsythia</i>	U1	14	14.3	1	7.0	3	21.4	0.50	0.29	0.49
	U6	14	35.7	7	50.0	7	50.0	0.35	0.65	0.35

* statistically significant at p<0.05

U1 – the right upper incisor; U6 – the right upper first molar

Table 3. Frequency of periodontopathogens in subgingival plaque at three times: before appliance removal (T1), one month after the appliance removal (T2), and three months after the removal (T3)

Group B		T1		T2		T3		p-value		
Microorganism	Tooth	N	%	N	%	N	%	T1 vs. T2	T2 vs. T3	T1 vs. T3
<i>Aggregatibacter actinomycetemcomitans</i>	U1	19	31.5	3	15.8	5	26.3	0.22	0.35	0.49
	U6	19	36.8	4	21.1	7	36.8	0.24	0.24	0.63
<i>Porphyromonas gingivalis</i>	U1	19	5.2	1	5.2	0	0	0.76	0.50	0.50
	U6	19	0	1	5.2	0	0	0.49	0.50	/
<i>Prevotella intermedia</i>	U1	19	26.3	6	31.6	7	36.8	0.49	0.49	0.36
	U6	19	31.6	8	42.1	5	26.3	0.37	0.25	0.49
<i>Tannerella forsythia</i>	U1	19	15.7	4	21.1	4	21.1	0.49	0.65	0.49
	U6	19	68.4	4	21.1	2	10.5	0.004*	0.33	0.0003*

* statistically significant at p<0.05

U1 – the right upper incisor; U6 – the right upper first molar

RESULTS

The frequencies of 4 analyzed bacteria at U1 and U6 sample sites in T1, T2 and T3 times of groups A and B are shown in Tables 2 and 3.

In patients with the placement of fixed appliance (group A), the frequency of *P. gingivalis* was decreased significantly, from T1 to T2 time, on both sample sites, the upper incisor ($p=0.049$) and the upper first molar ($p=0.008$), and for U1 from T1 to T3 time ($p=0.048$). Contrarily, in the same group significant changes in frequency of *A. actinomycetemcomitans*, *P. intermedia*, and *T. forsythia* were not noticed at U1 and U6 sites, from T1 to T3 time.

In patients with appliance removal (group B), only the frequency of *T. forsythia* was significantly reduced with time at U6 site (T1 vs. T2, $p=0.004$; T1 vs. T3, $p=0.0003$). No significant difference in frequency was registered for all other analyzed bacteria, from T1 to T3.

In the group A the following presence of all analyzed bacteria, comparing T1 and T3 recording time, the frequency of unchanged pattern (--) for U1 and U6 was 46% and 29%, respectively; the frequency of unchanged pattern (++) for U1 and U6 was 14% and 32%, respectively. A changed pattern (+) was observed in 18% for U1 and 16% for U6 sites. A changed pattern (+-) was noticed in 21% for U1 and 23% for U6 (Graph 1).

In the group B the frequency of all analyzed bacteria, comparing T1 and T3 at unchanged pattern (--), for U1 and U6, was 67% and 60%, respectively; the frequency of unchanged pattern (++), for U1 and U6 was 8% and 13%, respectively. A changed pattern (+) was seen in 13% for U1 and 5% for U6. A changed pattern (+-) was found in 12% for U1 and 21% for U6 (Graph 2).

DISCUSSION

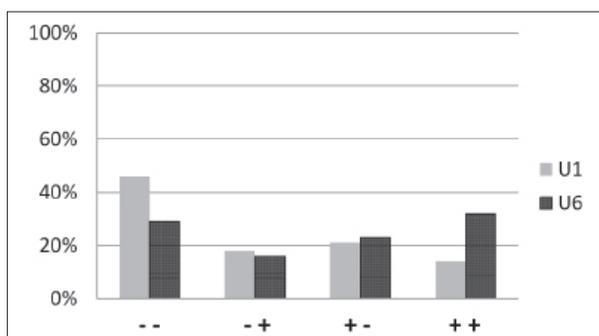
The inflammatory reaction of gingival tissues can be often detected among patients with fixed orthodontic appliances as a result of inadequate oral hygiene. The results of many studies about the effects of orthodontic therapy on gingival and periodontal health are rather inconsistent. Some of

them showed that subgingival microflora was changed in patients with an orthodontic appliance [5, 6], while others showed that those changes were reversible and had no significant effect on later periodontal health [7].

For periodontal microbial identification the most commonly used methods are cell culturing, PCR, and immunologically analysis. The cell culturing method is time consuming, expensive and may fail to grow some important organisms, while the PCR method shows greater sensitivity and specificity compared with other techniques [13]. Over the recent years, by using the PCR method most investigations have been directed to estimation of periodontopathogens during orthodontic therapy; however, there is still a lack of literature data about alterations of bacterial status after therapy [14, 15]. In our study PCRs were used to detect 4 anaerobes: *T. forsythia*, *A. actinomycetemcomitans*, *P. gingivalis*, and *P. intermedia* that are assumed as the main etiological factor for the development of periodontal disease [16].

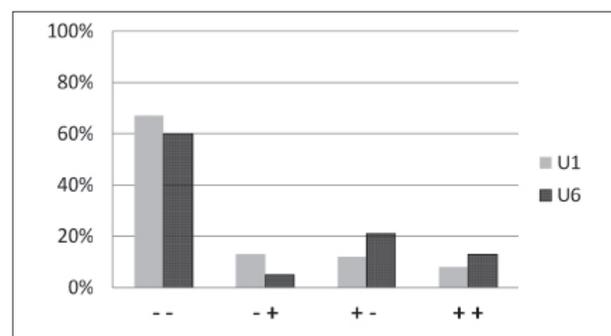
In the reports of Socransky et al. [16] two anaerobes, *T. forsythia* and *P. gingivalis*, have been categorized as the "red complex" species, which is related to the severity of periodontitis, while *A. actinomycetemcomitans* and *P. intermedia* are categorized as secondary risk factors involved in periodontal tissue destruction. According to data, *A. actinomycetemcomitans* is anaerobe linked with juvenile periodontitis, while *P. gingivalis* is found in normal microflora of the oral cavity, and has an important role in the etiology of adult periodontitis [17, 18]. In the study of Griffen et al. [19] applying a specific PCR assay, *P. gingivalis* was detected in only 25% of healthy subjects, and in higher rate (79%) of patients with periodontitis. Anyway, as known all four bacteria analyzed in our study may have synergistic effect in destroying periodontal tissues, as reported in investigation by Ashimoto et al. [13].

In the first of tested group (A) with patients at the beginning of orthodontic therapy; where we compared the frequency of sites positive for each bacteria in times, T1 (before placement), T2 (one month after), and T3 (three months after), no statistically significant difference was registered between recorded times for *A. actinomycetemcomitans*, *P. intermedia*, and *T. forsythia* (Table 2).



Graph 1. Group A: Comparison of subgingival microorganisms in each site at T1 (before placement of fixed appliance) and T3 (3 months after placement of the appliance)

(--) – microorganisms did not exist at T1 and T3; (+) – microorganisms did not exist at T1 but appeared at T3; (+-) – microorganisms existed at T1 but disappeared at T3; (++) – microorganisms existed at T1 and T3; U1 – the right upper incisor; U6 – the right upper first molar



Graph 2. Group B: Comparison of subgingival microorganisms in each site at T1 (before removal of fixed appliance) and T3 (3 months after removal of the appliance)

(--) – microorganisms did not exist at T1 and T3; (+) – microorganisms did not exist at T1 but appeared at T3; (+-) – microorganisms existed at T1 but disappeared at T3; (++) – microorganisms existed at T1 and T3; U1 – the right upper incisor; U6 – the right upper first molar

Only the frequency of *P. gingivalis* on U1 was significantly decreased in T2 and T3 compared to T1 time, and on U6 between T1 and T2, although we expected that it would be increased. As in our study, Liu et al. [10] showed the decrease of *P. gingivalis* during the first 3 months after the placement of the appliance, although the sampling sites were different. Similar to our data, in one of the first investigations of gingival changes of oral bacteria in patients during orthodontic treatment, Diamanti-Kipiotti et al. [20] registered a decreasing rate of bacteria (*B. intermedius* and *A. odontolyticus*) 4 months after the placement of brackets. Opposite results were obtained by Ristic et al. [8] reports involving clinical investigation which showed increased values of *P. gingivalis* and *F. nucleatum*, 3 months after placement applicants, but also their decrease 6 months after the beginning of orthodontic therapy.

As noted in our study, the absence of variation in frequencies for three anaerobes and the decreasing rate of *P. gingivalis* during 3 months from the beginning of orthodontics treatment might be explained in two ways. Namely, after the placement of appliances, new retentive places around the brackets could be formed, where the amount of supragingival plaque and aerobic bacteria might be increased, while anaerobes could be reduced and plaque composition modified. Also, one of the reasons for this microbiological status regarding anaerobes might be that the patients followed our instructions about oral hygiene in the first months of the orthodontic therapy. First 3 months of our treatment was the period when patients were highly motivated to take more care about dental hygiene, and as the consequence the presence of analyzed bacteria did not change significantly during this time. Interestingly, the study of Van Gastel et al. [4] showed a decrease of bacteria after bonding in the recording time (from week 18 to week 36).

As well known, the presence of some oral anaerobes might be associated with the hormonal level, especially during pregnancy and puberty [21]. Since the average age of our female patients was 19.2 years, microbiological changes can be probably related to individual dental hygiene habits. However, as shown in study by Shourie et al. [22], hormones may have a negligible effect on clinically healthy periodontium.

Additional data analyses confirmed our results of no significant changes of bacterial value during the period of therapy. Moreover, in the group A, summarizing the presence of all bacteria, either on U1 or U6 site, the patients with unchanged (--) / (++) patterns had higher values and also a changed pattern (+-) was noticed in higher percentage than in those with (-+) (Graph 1).

Also, in the second tested group (B), after the removal of the orthodontic appliance microbiological changes were followed at three different times: the time of appliance removal (T1), after one month (T2) and after three months (T3). In our study, only a decreasing of frequency for *T. forsythia* on U6 from T1 to T2, and T1 to T3 was noticed, with statistical significance. The same decreasing trend of *T. forsythia* on U1 through the time was not observed. Opposite to those results, other analyzed anaerobes showed no significant changes during the three-month period (Table 3). Similar to our results, in a report of Choi et al. [9] no statistical significant decrease of the same bacterial species was evaluated after the removal of orthodontic appliance. Opposite to our data, Sallum et al. [14] showed a significant reduction of anaerobes after the removal of the orthodontic appliance. Those contradictory results might be explained by different sampling sites, applied techniques, individual oral hygiene habits, and the use of prophylactic measures. Moreover, similar to our obtained data related to *T. forsythia*, Liu et al. [10] observed a reduced frequency of patients with *P. gingivalis* after 3 months from the removal of appliances. Anyway, as in our study, the same trend toward the reduction of bacteria was noticed in reports of other authors, especially on U6 after appliances removal [9, 10, 14].

Also, in the group B U6 showed a higher reduction of all bacteria, from T1 to T3 time than U1, so it might be suggested that U6 could be more relevant for the following microbiological changes. Analyzing the overall frequency in the group B, unchanged pattern (--) had the highest value, but the patients with changed pattern (+-) were present in a greater percentage than those with (-+) and (++) (Graph 2).

CONCLUSION

Placement of fixed appliances may have an impact on subgingival microflora, but in the first months after the placement of the appliance those changes were not significant, probably due to good oral hygiene. After the removal of fixed appliance the trend of decreased anaerobic bacteria was noticed. However, how long it takes to return to the preorthodontic composition of subgingival microflora remains to be seen.

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Промене субгингивалне микрофлоре након поставке и уклањања фиксних ортодонских апарата

Марија Живковић Сандић¹, Бранка Поповић², Јелена Чаркић², Нађа Николић², Бранислав Глишић¹

¹Клиника за ортопедију вилица, Стоматолошки факултет, Универзитет у Београду, Београд, Србија;

²Институт за хуману генетику, Стоматолошки факултет, Универзитет у Београду, Београд, Србија

КРАТАК САДРЖАЈ

Увод Поставка фиксних ортодонских апарата може довести до повећаног нагомилавања плака и промена у субгингивалној микрофлори.

Циљ рада Циљ овог рада био је да се испитају промене субгингивалне микрофлоре након поставке и уклањања фиксних ортодонских апарата применом реакције ланчаног умножавања молекула ДНК (енгл. *polymerase chain reaction – PCR*).

Методе рада Студија је обухватила 33 пацијента која су сврстана у две групе (А и Б). Узорци плака су узети из субгингивалног простора десног горњег централног секутића (У1) и десног горњег првог кутњака (У6). У групи А узорци су узимани пре поставке фиксног апарата (Т1), месец дана после поставке (Т2) и три месеца од поставке (Т3). У групи Б узорци су узимани пре уклањања апарата (Т1), месец дана после уклањања (Т2) и три месеца након уклањања (Т3). Применом методе *PCR* анализирано је постојање микроорганизама:

Porphyromonas gingivalis, *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia* и *Prevotella intermedia*.

Резултати У групи А учесталост *P. gingivalis* показала је статистички значајно смањење на оба зуба (У1: $p=0,049$; У6: $p=0,008$) у временском интервалу од Т1 до Т2. Уочено је и статистички значајно смањење заступљености ове бактерије на зубу У1 у интервалу од Т1 до Т3 ($p=0,048$). У групи Б само се учесталост *T. forsythia* статистички значајно смањила на зубу У6 у интервалу од Т1 до Т2 ($p=0,004$) и од Т1 до Т3 ($p=0,0003$). Учесталости осталих бактерија у обе групе испитаника нису показале статистички значајне промене.

Закључак Поставка фиксних апарата може да утиче на састав субгингивалне микрофлоре, али у првим месецима након поставке и уклањања апарата углавном нису уочене статистички значајне промене, вероватно због добре оралне хигијене.

Кључне речи: ортодонски апарат; бактерије; *PCR*