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ORIGINAL ARTICLE

Detection of *Aggregatibacter actinomycetemcomitans* and red complex bacteria from supragingival and subgingival plaque samples of healthy individuals before orthodontic treatment - A clinical and microbiological correlation

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Abstract

Background: Oral microbiota has an ecologically diverse microbial population. A disruption in the harmonious relationship between host and flora due to the placement of fixed orthodontic appliance might result in the initiation of periodontal diseases. The red complex organisms and Aa are known periodontal pathogens. Hence, investigating the potential for periodontal disease in patients before initiating orthodontic treatment would be beneficial and will help assess measures to be taken to maintain oral hygiene.

Materials and Methods: A total of 75 periodontally healthy individuals (42 males and 33 females) in the age group of 15–25 years with an indication for orthodontic treatment were included. The gingival index (GI) and plaque index (PI) were assessed for every patient. Supra- and Subgingival plaque samples were collected using sterile Gracey curettes. DNA extraction was done using modified proteinase K method. Multiplex polymerase chain reaction (PCR) was performed to detect selected microorganisms. Species-specific primers targeting 16SrRNA gene were used. The data were analyzed using ANOVA and *post hoc* Bonferroni test for the presence of a combination of microorganisms and were correlated to clinical parameters.

Results: The occurrence of a combination of microorganisms was significantly higher in subgingival plaque samples ($P < 0.05$) and was significantly correlated to the GI and PI ($P < 0.05$).

Conclusion: The rate of the occurrence of microorganisms was higher in subgingival plaque samples as compared to supragingival plaque. The combination of the occurrence of microorganisms in subgingival plaque samples was significantly correlated to clinical parameters such as GI and PI.

Introduction

The oral microbiota has an ecologically diverse microbial population. Oral health and disease depend on the interplay between the host and oral microbial community.[1] A disruption in this harmonious relationship between the host and flora either due to change in the pH of saliva, malalignment of the teeth, poor quality restorations, and diet/placement of intraoral appliance causes conversion of these organisms from commensal/opportunistic pathogens to the initiation of oral diseases including caries and periodontitis.[2-5] Several investigators have shown that periodontal diseases are associated with a consortium of organisms rather than just individual pathogens of periodontal sites.[6,7] Microbial complexes are repeatedly found together in subgingival biofilm with and without periodontal disease.[6] *Aggregatibacter actinomycetemcomitans* (Aa) and *Porphyromonas gingivalis* (Pg) are known periodontal pathogens.[8,9]

The impact of microbial community on shifting the balance from health to disease cannot be understood without a comprehensive view of a healthy community. Placement of fixed orthodontic appliance hinders maintenance of optimal oral

hygiene, thereby resulting in accumulation of dental plaque and subsequent gingival inflammation and periodontal diseases.[10-16] The formation of biofilms can exacerbate pre-existing periodontal diseases and cause enamel decalcification, affecting patients undergoing orthodontic treatment. Hence, investigating the potential for disease in patient during orthodontic treatment would be beneficial and would add to the knowledge in the field of oral diseases.

Culture is a traditional method for the detection of several microorganisms. However, all organisms cannot be detected through this method. Furthermore, it requires time and is labor intense. On the other hand, polymerase chain reaction (PCR) is sensitive, specific, and less time-consuming. Multiplex PCR is considered more effective for organism detection because of its versatility, high specificity, and sensitivity to detect multiple organisms.

Therefore, the present study was aimed to estimate the occurrence of the red complex organisms including Pg, *Treponema denticola* (Td), *Tannerella forsythia* (Tf), and Aa and their relative abundance in dental plaque in young healthy patient seeking orthodontic treatment using multiplex PCR.

Materials and Methods

The present study included a total of 75 periodontally healthy individuals (42 males and 33 females) in the age group of 15–25 years visiting the OPD of the Department of Orthodontics of our institution. The present study is a part of the research grant provided by Rajiv Gandhi University of Health Sciences (RGUHS), Bengaluru. The study was commenced after obtaining permission from the Institutional Review Board.

Individuals with healthy systemic condition, periodontal probing depth of ≤3 mm with no history of periodontal treatment in the past 6 months, and non-smokers who showed no evidence of bone loss on an OPG were included in the study. Patients with any systemic diseases/medically compromised and who were on medications such as antibiotics and antimicrobials within 3 months before the sampling were excluded from the study. Pregnant and lactating women as well as patients who have undergone periodontal therapy within 3 months before sampling also were not included in the study. Individual who met all the above-mentioned inclusion and exclusion criteria were considered for the study.

After obtaining the written informed consent from the each participant, a complete oral examination for both the soft and hard tissues was performed. The gingival index (GI) and plaque index (PI) were recorded for each patient. The plaque samples were collected using sterile Gracey Curettes. Supragingival plaque was collected by stripping of the plaque from the minimum of six different teeth ranging from incisor to first molars, and subgingival plaque was collected after all the supragingival plaque was removed from the selected tooth surface. These samples were transferred immediately to the transport media, reduced transport fluid, and transported to the Central Research Laboratory of our institution.

DNA from the supragingival and subgingival plaque sample was extracted within 48 h of specimen collection. This was carried out using "Modified Proteinase- K method." The plaque samples were vortexed and centrifuged at 5000 rpm for 5 min. The supernatant was discarded, and then plaque was suspended in fresh TE (Tris–EDTA) buffer. The above step was repeated for 2–3 times, and bacterial cells were lysed using Lysis buffer I containing 10 mM Tris buffer, 1% triton-100, and 1 mM EDTA. This was followed by adding lysis II buffer containing 50 mM Tris hydrochloride, 50 mM KCl, 2.5 mM magnesium chloride, and 0.45% Nonidet P40. Protein contaminants were degraded using Proteinase K enzyme (10 mg/mL). The samples were kept at 60°C for 2 h and then at 100°C in boiling water bath for 10 min for enzyme deactivation. Further, they were centrifuged at 5000 rpm for 3 min, and supernatant containing DNA was collected in sterile tubes and preserved at −20°C till the PCR was performed.

Multiplex PCR was performed to detect Aa, Pg, Tf, and Td. Species-specific primers targeting 16S rRNA gene of each bacterium were used in this study. The list of primers used is given in Table 1.

PCR was carried out in a total volume of 25 μL containing 3 μL of DNA template of approximately 100 μg/mL concentration. PCR master mix was prepared using multiplex PCR Kit (Qiagen, New Delhi, India) which contained 1.5 mM

Table 1: List of primers

Primer pairs 5'-3'	Amplification length
Actinobacillus actinomycetemcomitans GCT AAT ACC GCG TAG AGT CGG ATT TCA CAC CTC ACT TAA AGG T	443 bp
Tannerella forsythensis (Bacteroides forsythus) GCG TAT GTA ACC TGC CCG CA TGC TTC AGT GTC AGT TAT ACC T	641 bp
Campylobacter rectus TTT CGG AGC GTA AAC TCC TTT TC TTT CTG CAA GCA GAC ACT CTT	598 bp
Fusobacterium nucleatum GAA GAA ACA AAT GAC GGT AAC AAC GTC ATC CCC ACC TTC CTC CT	705 bp
Eikenella corrodens CTA ATA CCG CAT ACG TCC TAA G CTA CTA AGC AAT CAA GTT GCC C	688 bp
Porphyromonas gingivalis AGG CAG CTT GCC ATA CTG CG ACT GTT AGC AAC TAC CGA TGT	404 bp
Prevotella intermedia AAC GGC ATT ATG TGC TTG CAC CTC AAG TCC GCC AGT TCG CG	589 bp
Treponema denticola TAA TAC CGA ATG TGC TCA TTT ACA T TCA AAG AAG CAT TCC CTC TTC TTC TTA	316 bp
Ubiquitous primer GAT TAG ATA CCC TGG TAG TCC AC CCC GGG AAC GTA TTC ACC G	602 bp

magnesium chloride, 200 μM of mixture of deoxynucleoside triphosphates, ×1 PCR buffer containing 10 mM Tris-HCl, pH 8.0, 50 mM potassium chloride,and 2.5 unit of hot start Taq DNA polymerase. Samples were kept in a verity thermal cycler (Applied Biosystems, California, United States). The thermal cycling condition was as follows: Initial denaturation was done at 95°C for 15 min followed by 35 cycles of denaturation at 94°C for 1 min and extension at 72°C for 1 min. Final extension was carried out for 10 min. Following PCR, amplified products were detected by separating the samples on 2% agarose gel electrophoresis in Tris-Acetate-EDTA buffer for 2 h at 70V. The gel was stained with ethidium bromide (0.5 μg/mL). The gel was visualized under UV transilluminator [Figure 1] (Gel Doc, major science). The molecular size of each band was determined by comparing with ready to use "Next Gen"100bp DNA ladder (Genetix Biotech Asia Pvt Ltd, New Delhi, India).

Statistical analysis was done using SPSS software version 17.0. $P < 0.05$ is considered statistically significant and $P > 0.05$ is statistically non-significant.

Results

Of the 75 samples collected, 42 were males and 33 females in the age range of 15 years25 years.

The presence or absence of individual organisms was assessed for supra and subgingival plaque samples. The percentage of the occurrence of individual organisms in supragingival and subgingival plaque was estimated, and Fisher's exact test was applied to see for statistical significance [Table 2]. Subgingival plaque showed slightly higher percentage of occurrence of organisms when compared to supragingival plaque samples. However, the comparison between the two was statistically insignificant except for Tf, which showed a statistically significant difference [Table 2].

On comparison between males and females for the occurrence of selected organisms in supragingival plaque samples, females showed a slightly higher prevalence for Aa (21.2%), Pg (27.27%), and Tf (15.15%), whereas Td (9.09%) was almost same in both males and females. In subgingival on comparison between males and females for the occurrence of the organisms, Aa showed almost the same prevalence in both males and females (21.4% and 21.2%, respectively). Pg was slightly higher in females as compared to males (30.3% and 28.5%, respectively). Tf and Td were higher in males (23.8% and 26.1%, respectively) when compared to females (21.2% and 21.2%, respectively).

On comparison between the occurrence of selected microorganisms between supra- and subgingival plaques between males and females, subgingival plaque samples showed higher occurrence of microorganisms as compared to supragingival samples.

The data were analyzed using ANOVA to see for the presence of a combination of organisms Aa-Pg, Aa-Tf, Aa-Td, Pg-Tf, and Pg-Td and were correlated with clinical parameters. For the values that were statistically significant ($P < 0.05$), the data were subjected to *post hoc* Bonferroni test.

Table 2: Prevalence of individual organisms in supra and sub gingival plaque samples

Organism	Plaque (%)		*P* value (<0.05)
	Supragingival	Subgingival	
Aa	14 (45.2)	17 (54.8)	0.687, NS
Pg	15 (39.5)	23 (60.5)	0.188, NS
Td	12 (41.4)	17 (58.6)	0.409, NS
Tf	6 (25.0)	18 (75.0)	0.013, S

P<0.05 considered significant. Aa: *Actinobacillus actinomycetemcomitans*, Pg: *Porphyromonas gingivalis*, Td: *Treponema denticola*, Tf: *Tannerella forsythia*

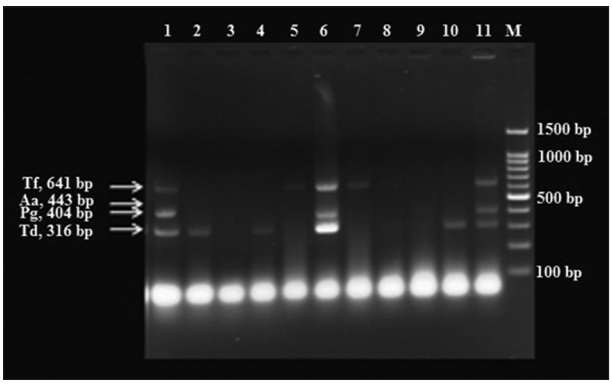

Figure 1: Positive bands in gel electrophoresis under UV light for *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*

When combination of the occurrence of microorganisms Aa-Pg, Aa-Tf, Aa- Td, Pg-Tf, and Pg-Td was assessed, all of these combinations of occurrence were higher in subgingival plaque as compared to supragingival plaque samples [Table 3], but it was statistically insignificant.

When combination of the occurrence of microorganisms in subgingival plaque samples was correlated with clinical parameters such as GI and PI, it was observed that, for all the combinations, it was statistically significant [Table 4].

Discussion

One of the major concerns during orthodontic treatment is conserving the integrity of periodontal tissues. The placement of orthodontic appliances creates plaque retentive areas[10-16] posing difficulty to maintain oral hygiene, thereby acting as a risk factor for periodontal diseases. Changes in the oral microbiota that leads to periodontal breakdown during orthodontic treatment are mainly associated with a change in biofilm equilibrium[17] and increase in plaque accumulation,[18] causing an increase in periodontopathogenic species.[19-21] Studies have conflicting observations where patients with average oral hygiene did not pose any change in gingival health. Numerous studies have investigated the effect of orthodontic treatment on periodontal health, in which most of them concluded that changes in the gingival health produced during orthodontic treatment

Table 3: Percentage combinations of the occurrence of Aa Pg, Aa-Tf, Aa-Td, Pg-Tf, and Pg-Td in plaque samples

Aa-Pg	Count %	Supra gingival (%)	Sub gingival (%)	Test of significance; Chi-square test
	None present	51 (52.6)	46 (47.4)	Pearson Chi-square 2.535, df – 2, *P*=0.282, NS
	Either Aa or Pg present	19 (51.4)	18 (48.6)	
	Both Aa and Pg present	5 (31.3)	11 (68.8)	
Aa-Tf	None present	58 (54.7)	48 (45.3)	Pearson Chi-square 3.974[a], df – 2, *P*=0.137, NS
	Either Aa or Tf present	14 (42.4)	19 (57.6)	
	Both Aa and Tf present	3 (27.3)	8 (72.7)	
Aa-Td	None present	52 (69.3)	47 (62.7)	Pearson Chi-square 1.348[a], df – 2, *P*=0.510, NS
	Either Aa or Td present	20 (26.7)	22 (29.3)	
	Both Aa and Td present	3 (4.0)	6 (8.0)	
Pg-Tf	None present	57 (76.0)	43 (57.3)	Pearson Chi-square 6.644[a], df – 2, *P*=0.036, S
	Either Pg or Tf present	15 (20.0)	23 (30.7)	
	Both Pg and Tf present	3 (4.0)	9 (12.0)	
Pg-Td	None present	51 (68.0)	45 (60.0)	Pearson Chi-square 4.169[a], df – 2, *P*=0.124, NS
	Either Pg or Td present	21 (28.0)	20 (26.7)	
	Both Pg and Td present	3 (4.0)	10 (13.3)	

P<0.05 considered significant. Aa*: Actinobacillus actinomycetemcomitans*, Pg: *Porphyromonas gingivalis*, Td: *Treponema denticola*, Tf: *Tannerella forsythia*

Table 4: Correlation of clinical parameters with presence/absence of combination of organisms in subgingival plaque samples

Plaque	Clinical Parameters	N	Mean	SD	95% Confidence Interval for Mean		ANOVA	*Post hoc* bonferroni tests		
					Lower bound	Upper bound		None versus either	None versus both	Either versus both
Subgingival	GI									
	None	46	0.5	0.69	0.29	0.71	f-ratio ; 30.68, *P*: <0.001, S	<0.001, S	<0.001, S	>0.05, NS
	Either Aa or Pg	18	1.61	0.5	1.36	1.86				
	Both Aa and Pg	11	1.73	0.47	1.41	2.04				
	Total	75	0.95	0.84	0.75	1.14				
	Plaque index									
	None	46	1.7	0.41	1.58	1.82	f-ratio ; 31.49, *P*: <0.001, S	<0.001, S	<0.001, S	>0.05, NS
	Either Aa or Pg	18	2.43	0.38	2.24	2.61				
	Both Aa and Pg	11	2.5	0.4	2.23	2.77				
	Total	75	1.99	0.54	1.87	2.12				
Subgingival	GI									
	None	48	0.5833	0.76724	0.3606	0.8061	f-ratio ; 18.55, *P*:<0.001, S	<0.001, S	<0.001, S	>0.05, NS
	Either Aa or Tf	19	1.5789	0.50726	1.3345	1.8234				
	Both Aa and Tf	8	1.625	0.51755	1.1923	2.0577				
	Total	75	0.9467	0.83655	0.7542	1.1391				
	Plaque index									
	None	48	1.7354	0.43588	1.6088	1.862	f-ratio ; 24.23, *P*: <0.001, S	<0.001, S	<0.001, S	>0.05, NS
	Either Aa or Tf	19	2.4474	0.39633	2.2563	2.6384				
	Both Aa and Tf	8	2.45	0.42762	2.0925	2.8075				
	Total	75	1.992	0.54295	1.8671	2.1169				

(Contd...)

Table 4: *Continued*

Plaque	Clinical Parameters	N	Mean	SD	95% Confidence Interval for Mean		ANOVA	*Post hoc* bonferroni tests		
					Lower bound	Upper bound		None versus either	None versus both	Either versus both
Subgingival	GI									
	None	47	0.5106	0.68754	0.31	0.71	f-ratio ; 30.94, *P*: <0.001, S	<0.001, S	<0.001, S	>0.05, NS
	Either Aa or Td	22	1.6818	0.47673	1.47	1.89				
	Both Aa and Td	6	1.6667	0.5164	1.12	2.21				
	Total	75	0.9467	0.83655	0.75	1.14				
	Plaque index									
	None	47	1.7149	0.43036	1.59	1.81	f-ratio ; 28.98, *P*: <0.001, S	<0.001, S	<0.001, S	`>0.05, NS
	Either Aa or Td	22	2.4818	0.33329	2.33	2.52				
	Both Aa and Td	6	2.3667	0.49666	1.85	2.87				
	Total	75	1.992	0.54295	1.87	2.12				
Subgingival	GI									
	None	43	0.4651	0.702	0.25	0.68	f-ratio ; 30.60, *P*: <0.001, S	<0.001, S	<0.001, S	>0.05, NS
	Either Pg or Tf	23	1.5217	0.51075	1.3	1.74				
	Both Pg and Tf	9	1.7778	0.44096	1.44	2.12				
	Total	75	0.9467	0.83655	0.75	1.14				
	Plaque index									
	None	43	1.6767	0.41681	1.55	1.81	f-ratio ; 31.74, *P*: <0.001, S	<0.001, S	<0.001, S	>0.05, NS
	Either Pg or Tf	23	2.3652	0.3588	2.21	2.52				
	Both Pg and Tf	9	2.5444	0.42753	2.22	2.87				
	Total	75	1.992	0.54295	1.87	2.12				
Subgingival	GI									
	None	45	0.44	0.62	0.26	0.63	f-ratio ; 43.63, *P*: <0.001, S	<0.001, S	<0.001, S	>0.05, NS
	Either Pg or Td	20	1.7	0.47	1.48	1.92				
	Both Pg and Td	10	1.7	0.48	1.35	2.05				
	Total	75	0.95	0.84	0.75	1.14				
	Plaque index									
	None	45	1.68	0.41	1.56	1.81	f-ratio ; 34.66, *P*: <0.001, S	<0.001, S	<0.001, S	>0.05, NS
	Either Pg or Td	20	2.49	0.33	2.33	2.64				
	Both Pg and Td	10	2.39	0.43	2.08	2.7				
	Total	75	1.99	0.54	1.87	2.12				

P<0.05 considered significant. Aa: *Actinobacillus actinomycetemcomitans*, Pg: *Porphyromonas gingivalis*, Td: *Treponema denticola*, Tf: *Tannerella forsythia*

were transient with no permanent damage to the periodontal tissues.[3,5] It was rather the level of oral hygiene that had a direct bearing on the periodontal health during orthodontic treatment. For years, it has been suggested that bacterial plaque is the major etiological factor in the initiation and progression of gingivitis and periodontitis.[22] Placement of orthodontic bands on molar teeth positioned subgingivally acts as a nidus for the accumulation of plaque around gingival crevice. Regardless of maintaining good oral hygiene regime, majority of the patients develop gingivitis within a short time of placement of fixed appliance,[3,5,23] thus making it necessary to know the prevalence of the organisms most commonly associated with periodontitis in young healthy individuals. Hence, the current study aimed to see the occurrence of Aa and red complex organisms in young patients before initiation of orthodontic treatment and their correlation with clinical parameters.

In the present study, we found subgingival plaque showing slightly higher percentage for Aa, Pg, Td, and Tf as compared

to supragingival plaque, suggesting that red complex organisms Pg, Td, and Tf have a strong relationship between each other in subgingival plaque samples.[6] Tf and Pg have been detected more frequently in higher numbers in deeper pockets[24] which can be correlated to the observation of the study where the combination of occurrence of Tf and Pg showed significant correlation with GI and PI. A strong correlation between Pg and Td was observed in subgingival plaque samples,[25] and in our study, we found similar observation showing a significant correlation of Pg and Td combination with PI and GI.

In the present study, Pg was seen in higher concentration in females when compared to males which is in consensus to the review findings of Effie.[26] The author suggested that the microbial gender variations observed were mainly based on innate immune response of individuals and suggested that the host microbial hypothesis in the pathogenesis of periodontal diseases between males and females needs further evaluation. However, in the present study, we definitely found some difference in the prevalence of the selected microorganisms between males and females.

The occurrence of Aa-Pg in combination showed significant correlation with PI and GI and also the prevalence was high in subgingival plaque compared to supragingival plaque, suggesting that Aa and Pg are putative periodontal pathogens. This finding is similar to the findings of several other researches.[20,21,27-30] Studies have also isolated red complex and Aa together in the subgingival biofilm of subjects with and without periodontal diseases and these organisms which form a biofilm are considered to be the most pathogenic microbial complex.[6]

Clinical and microbiological effects on fixed orthodontic appliance showed that placement of orthodontic bands in children in the absence of optimal oral hygiene will result in increased pocket probing depth along with shift in the microbial composition of supra- and subgingival plaque.[5] In our study, we found that the prevalence of red complex and Aa was more in subgingival plaque sample which might have an influence on disease progression once orthodontic treatment is started.

Few studies have also showed a minimal increase in plaque levels in subjects who were taking active orthodontic treatment. However, during orthodontic treatment with fixed appliances, an increase in gingivitis score and PI[20,21,31-33] is almost always encountered which may be partly attributed to mechanical injury caused by subgingival placement of orthodontic bands or increased plaque retentive areas around orthodontic brackets.

In a study by Diamanti *et al*.,[13] they did not observe any change in the supragingival plaque formation in patients receiving orthodontic treatment. In contrast to this findings, we found red complex and Aa in healthy individuals before initiation of orthodontic treatment, which suggests that the individuals who have shown the presence of organisms are more prone to periodontal diseases on commencement of orthodontic treatment. Hence, a baseline assessment of known periodontal pathogens is of valuable aid.

In consensus with the findings of numerous studies, we too found that GI and PI were significantly associated with subgingival plaque microflora. These findings will help us assess the progression of associated factors during the course of orthodontic treatment, thereby providing knowledge to take necessary measures and oral hygiene regimen to avoid periodontal destruction.

Conclusion

In the present study, Aa, Pg, Tf, and Td, the known periodontal pathogens were present in both supra- and subgingival plaque samples of healthy patients with a slightly higher percentage in subgingival plaque samples. Even though these are normal commensals, placement of fixed orthodontic appliance provides areas for plaque retention stimulating change in oral environment that could lead to disease initiation and progression. Assessing the baseline plaque samples before orthodontic treatment will help in monitoring and assessing the clinical effects on periodontal health that will cater to educating the patient on the importance of maintaining oral protocol during the course of orthodontic treatment.

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