Biofilm Associated Microorganisms on Removable Oral Orthodontic Appliances in Children in the Mixed Dentition

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Objective: *Biofilms on removable orthodontic appliances act as reservoir of microorganisms, capable of modifying the environmental condition of oral cavity and are difficult to be removed with routine hygiene measures. The present investigation includes enumeration, identification and numerical analysis of different types of cultivable bacteria associated with the biofilms on removable orthodontic appliances.* ***Study design:*** *Removable appliances of 25 healthy children among the ages of 10 to 14 years were taken to measure the prevalence of biofilms and type of microorganisms. For isolation of microorganism from biofilms different types of selective and non-selective medium based on standard methods were used. The data were further analysed by using Kolmogorov-Smirnov test, one-sample t-test and Spearman rank correlation coefficient. The percentage frequencies of isolates were also calculated.* ***Results :*** *The survey revealed the presence of both multi-species and mono species biofilms on appliances, with Non-Streptococci, anaerobic bacteria, Streptococcus spp., members of the family Enterobacteriaceae and Lactobacillus spp. as a dominant microbial flora of biofilms. Bacilllus sp. and Candida sp. were isolated from one sample each. Significant positive and negative correlations were established among the species isolated from biofilms.* ***Conclusion*** *Higher prevalence of the members of the family Enterobacteriaceae were reported during this study, advocating an extra hygienic measure is essential for this age group while wearing acrylic orthodontic appliances in oral cavity.*

Keywords: *biofilms, orthodontic removable appliances, children, gingivitis.*

INTRODUCTION

Nowadays orthodontic treatment is adopted by wide section of society not only for the corrections of malocclusion but also improves mastication, speech and appearance, as well as overall health, comfort, and self-esteem.[1] Although the orthodontics appliance has many known benefits, these appliances are also associated with a number of damages and disorders of oral cavity.[2] Bjerklin *et al* [3] reported higher proximal caries progression on canines, premolars and molars among children treated with removable orthodontic appliances.

Oral cavity is a complex environment supporting more than 700 distinct bacterial species or phylotypes, of which over 50% are yet to be cultivated, residing specifically in diverse niches in the oral cavity and executing different roles.[34] Presence of orthodontic appliances in oral cavity alters the balanced ecosystem of oral microbiome; as it provides an additional retentive site for food, different physio-chemical environment and surfaces for adhesion and attachments of normal oral microflora. All these factor lead to changes in oral ecosystem by harbouring less commensal to more pathogenic strains.[5]

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Formation of biofilms by organisms growing in association with a surface is a very common phenomenon among microorganisms.[6] Biofilms are defined as microbial communities encased in a matrix of complex extracellular polymeric substance (EPS) and, exhibiting phenotypic features that differ with their planktonic forms.[7,8] Otitis media, osteomyelitis, native valve endocarditis, cystic fibrosis, chronic bacterial prostatitis, pneumonia etc. are some of the infections associated with biofolms, and the biofilms associated with the oral cavity implicated in gingivitis, periodontitis, dental caries, enamel scar, peri-implant infections and stomatitis.

The higher virulence of microorganisms associated with biofilms than its planktonic counterparts can be attributed to one or all the following factors: (i) formation of slime encased community which further act as a source of viable microorganism to its surroundings even causing blood stream infection, (ii) by releasing components toxic to host (iii) provision of persistors cells, and (iv) evasion from chemotherapeutic agents and host immune system.[9,10] It was previously been reported that almost all human microbial infections (>65%) involve biofilms formation.[9,11] Biofilms carries important clinical consequence as it act as a reservoir of infectious agent,[9,10,11] and hence the study of biofilms is not only important for prevention of infection and but also helpful in formulation of more efficacious strategies to control biofilms associated damages and disorders.

Despite several preventive measures have been taken to control biofilms formation on orthodontic appliances; the prevalence of biofilms related problems has remained high[12] especially in children's and immunocompromised patients. Abundant studies are there about biofilms in fixed orthodontic appliances, prosthetic devices and implants. But very few studies are available about

Table 1. One-sample Kolmogorov-Smirnov test and percentage frequency (%F) of the microorganism isolated from biofilms.

One-Sample Kolmogorov-Smirnov Test

	%F	Normal Parameters(a,b)		Most Extreme Differences			Kolmogorov-Smirnov Z	Asymp. Sig. (2-tailed)	Decision
		Mean	Std. Deviation	Absolute	Positive	Negative			
Anaerobic Bacteria	75	8,520	8,888	0.385	0.385	-0.240	1.156	0.138	Non Sig.
Enterobacteriaceae	91.67	2,507	3,923	0.362	0.362	-0.263	1.199	0.113	Non Sig.
Gram neg. Bacilli	33.33	285	179	0.162	0.145	-0.162	0.324	1.000	Non Sig.
Non-Streptococci	41.67	22,336	31,060	0.339	0.339	-0.241	0.757	0.615	Non Sig.
Streptococcus spp.	58.33	6,957	4,282	0.217	0.217	-0.147	0.574	0.897	Non Sig.
Lactobacillus spp.	83.33	4,584	8,624	0.346	0.346	-0.309	1.093	0.183	Non Sig.
Bacilllus sp.	8	10,400	Cannot be computed.						
Candida sp.	8	20	Cannot be computed.						
a. Test distribution is Normal.									
b. Calculated from data.									

biofilms on removable orthodontic appliances worn by children of mixed dentition age. Batoni *et al* [13] had studied the prevalence of *mutans streptococci* in particular in children with removable orthodontic appliances. Their study was not about the identification of bacteria associated with biofilms formation which might be responsible for variety of clinical problems.

The present study was constituted as some of the patients wearing removable orthodontic appliances presented with halitosis, gingivitis and palatal inflammation. These clinical problems moved authors to investigate the origin. In the present study, the microorganisms of biofilms associated with removable orthodontic appliances used to intercept developing malocclusion in mixed dentition age were analyzed.

MATERIALS AND METHOD

Twenty five removable orthodontic appliances of 25 healthy children, being treated in the department of Pediatric Dentistry, Modern Dental College and Research Centre, Indore, India were selected for the study. Exclusion criteria included the use of oral antimicrobials or antibiotics within past 3 months, the presence of fixed prosthesis, or significant systemic disease. Appliances were made up of self cured polymethylmethacrylate (PMMA) resins (Rapid Repair, India) and round stainless steel wire. These appliances were sent to the department of Microbiology, Modern Dental College and Research Centre, Indore, India to evaluate as the site of biofilms formation by the oral microbial community.

Immediately after the initial collection of appliances, the samples were processed for microbiological investigation. Each orthodontic appliance was first washed with sterile distilled water to remove non adherent cells from the surface of retainers. For quantitative estimation, retainers were placed in 10 mL. of Phosphate buffer saline (PBS) to remove and collect all the adherent cells, sonicated for five min. to disaggregate clumps, vortexed vigorously to maximize the recovery of microbial cells from biofilms. 50 µL of resulting cell suspensions were serially diluted and plated onto 90 mm. Petri plates containing Hichrome candida agar Medium (Hi Media, Mumbai, India), Eosin methylene blue Agar Medium (Hi Media, Mumbai, India), Hichrome UTI agar Medium (Hi Media, Mumbai, India), Tryptone Glucose Yeast Extract (TGYE) Agar Medium (Hi Media, Mumbai, India), Blood agar medium base (Hi Media, Mumbai, India) with 5% defibrinated blood, Thioglycolate agar medium(Hi Media, Mumbai, India), Lactobacillus MRS agar medium (Hi Media, Mumbai, India), Mitis Salivarius (MS) Agar with and without 15% sucrose plus bacitracin (0.2 units/ml) and Wilkins Chalgren agar Anaerobic Agar Base with Non Spore Anaerobic Supplement containing sodium pyruvate, menadione, hemin and nalidixic acid (Hi Media, Mumbai, India), all the media were prepared as per manufacturer instruction, for the enumeration of cultivated micro-organism associated with biofilms in terms of colony forming units (cfu) after adjusting with dilution factor.

In this study, identification of isolates (of sources and air) was done by using HiEnterobacteriaceae Identification Kit (Hi Media, Mumbai, India) and standard methods and manuals.[14,15,16] The identification of *Candida* species was conducted by culture characteristics on HiChrome *Candida* agar medium (HiMedia, Mumbai, India), assessing germ tube, chlamydospore formation and sugar assimilation patterns.[17]

The data were analysed by using SPSS (Statistical Package for Social Sciences for Windows, Chicago, Illinois, USA). To determine the relation existing among the cfu of different isolates the Spearman rank correlation coefficient (Spearman ρ) test was performed, to establish whether the data was normally distributed, the Kolmogorov-Smirnov test was performed in order to assess the goodness of fit; the test indicated ($P < 0.05$) that the results did not fit a normal distribution. and to test whether the sample comes from a population with a specified mean, $\mu 0$, or if there is a statistically significant difference between the observed mean in the sample and the hypothetical mean on the population ($\mu 0$) under $H0$ one-sample t-test was performed.[18] The percentage frequency (%F= No.of observations in which colony appear / Total no of observations recorded ×100) and average of isolates were also calculated.

Table 2. One-sample t-Test of the quantity of microorganism isolated from biofilms

One-Sample t-Test

	Test Value = 0					
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
Anaerobic Bacteria	2.876	8	0.021	8,520.000	1,687.83	15,352.17
Enterobacteriaceae	2.119	10	0.060	2,507.273	-128.53	5,143.08
Gram negative Bacilli	3.181	3	0.050	285.000	-0.09	570.09
Non-Streptococci	1.608	4	0.183	22,336.000	-16,229.99	60,901.99
Streptococcus	4.298	6	0.005	6,957.143	2,996.67	10,917.62
lactobacillus	1.681	9	0.127	4,584.000	-1,585.15	10,753.15

RESULTS

Out of 25 appliances investigated, 12 were reported positive for biofilms. Which were then evaluated for microbial composition. Figure-1 shows the composition in percentage of total viable cultivable bacteria of biofilms associated with orthodontic appliances. Colony forming units of cultivable bacteria isolated in descending order (Mean±Standard Deviation) were non-streptococci (includes gram negative aerobic cocci other than *Streptococcus* spp.) $2.2\pm3.1X10^6$, anaerobic bacteria (Includes gram negative and positive anaerobic bacilli and cocci) $8.5\pm8.9X10^5$, *Streptococcus* spp. $7.0\pm4.3X10^5$, members of the family *Enterobacteriaceae* $2.5\pm3.9X10^5$, Gram negative bacilli (includes glucose fermenting and non-fermenting aerobic and anaerobic species other than *Enterobacteriaceae*) $2.9\pm1.8\ X10^4$, and *Lactobacillus* spp. $4.6\pm8.6X10^5$, *Bacilllus* sp. (cfu-$10.4X10^5$), and *Candida* spp. (cfu-$2X10^3$). *Candida* spp. was positive only in one sample, therefore standard deviation and other statistical analyses were not performed for these two isolates.

Standard deviation higher than mean were observed in this statistical analysis shows higher degree of variance in the cfu of isolated species from the biofims obtained from the orthodontic appliances. However, the results of the Kolmogorov-Smirnov test showed that the data was normally distributed (Table-1). Percentage frequency of identified viable cultivable bacteria of biofilms associated with orthodontic appliances were (in descending order) the members of the family- *Enterobacteriaceae* 92%, *Lactobacillus* spp. 83%, anaerobic bacteria 75%, *Streptococcus* spp. 58%, non-streptococci 42%, other Gram negative bacilli 33% of frequency and *Bacilllus* sp. and *Candida* sp. 8.33% each (Table-1)

Out of 12 retainers evaluated, 11 retainers were harbouring multi-species biofilms consisting variety of micro-organisms; whereas, from one retainer mono-species biofilms were recovered consisting species of *Lactobacillus*. The correlation analysis revealed that there were positive correlation established among the species of *Lactobacillus* with anaerobic bacteria (p 0.982, significant at the 0.01 level), *Streptococcus* spp. with non-streptococci (p 1.00, significant at the 0.01 level) and there were negative correlation established among the species of Gram negative bacilli with non-streptococci (p -0.99, significant at the 0.05 level); members of the family *Enterobacteriaceae*, *Bacilllus* sp. and *Candida* sp. did not show any correlation with the other organism isolated from biofilms.

The student t-test revealed that, *Streptococcus* spp., anaerobic bacteria and other Gram negative Bacilli come from a population which normally participated in the formation of biofilms with a specified mean, its significant level were 0.005, 0.021 and 0.05 respectively and confidence interval of the difference does not contain zero except for other Gram negative bacilli. (Table-2)

DISCUSSION

The oral microbiome is influenced by various factors including food habit, aging, socio-economic status, dental hygiene measures as well as intra-oral prosthetic or orthodontic devices. These devices are one of the major factors governing shifting of complex commensal community of oral cavity towards source of pathogen by providing extra site of adhesion and attachment in the form of biofilms, thus acting as a reservoir of pathogens. The biofilms on intra-oral devices affect the appliance by corroding its surfaces which, not only modify the mechanical properties of these appliances but also increases the surfaces area where more microorganism could further be attached.[19] It is generally accepted that a shift in microbial composition is an important step in the progression of oral disease.[20] Biofilm that confers survival advantages to most of the pathogenic microorganism of humans is one of the well studied virulence factor of microorganism.[21]

According to Kolenbrander,[22] all oral bacteria have a capacity to adhere to other species of oral cavity, thus; the formation of multi-species biofilms in oral cavity and on appliances is inevitable. During present study, wide varieties of bacteria i.e. members of the family *Enterobacteriaceae*, *Lactobacillus* spp., anaerobic bacteria, *Streptococcus* spp., non-streptococci, Gram negative bacilli, *Bacilllus* sp. and *Candida* sp. were reported from biofilms associated with orthodontic appliances. Species of *Bacilllus* and *Candida* were recovered from one sample each showing candidal carriage. Presence of gut microflora in biofilms associated with orthodontic appliances are of serious concern for this age groups.

Batoni *et al* [13] also found higher number of mutans streptococci in children being treated with removable orthodontic appliance and stressed the importance of a careful monitoring of patients for risk of caries development.

Presence of *Candida* sp. identified in this study might be responsible for palatal stomatitis in our patients as seen in complete denture patients.[6] The prevalence was found very low perhaps because healthy individuals were taken and finding were similar

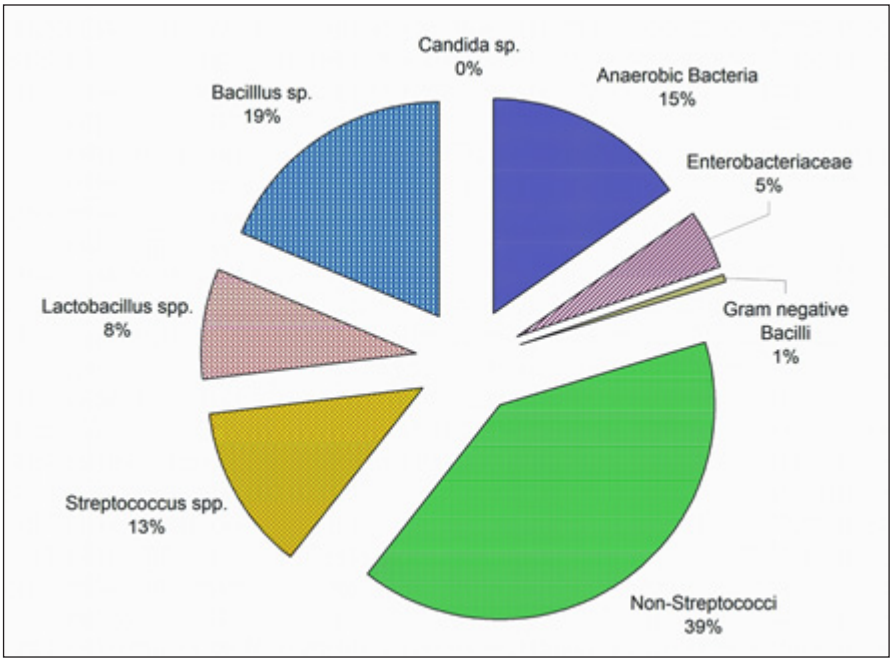

Figure 1. Average percentage of total type bacterial isolates from biofilms

to Hibino *et al* [4] who found that no healthy individual developed Candida infection though there was a trend that some non-Candida carriers converted to Candida carriers following the insertion of the appliances by unknown mechanism.

Previous studies reported higher prevalence of *Enterobacteriaceae* and *Pseudomonadaceae* in the oral cavity of different age group persons with and without orthodontics appliances[23,24] and on the tongue samples in children under 18 months.[25] According to Sumi *et al* acrylic bases act as a reservoir of respiratory pathogens and can be a risk factor for the pharyngeal colonization and aspiration pneumonia.[26] Increased counts of coliforms were also observed among individuals treated with fixed orthodontic appliances.[27] During present study, higher prevalence of the members of the family *Enterobacteriaceae* and Gram negative bacilli other than *Enterobacteriaceae* were reported from the biofilms associated with orthodontic appliances of children is in concord with previous findings. Goldberg *et al* [24] found potential association of *Enterobacteriaceae* with halitosis and its identification in this study could be related to halitosis in our patients.

The previous researchers while studying multi-species biofilms in oral cavity and on prosthetic devices also reported species of *Acinetobacter,*[28,29] anaerobic bacteria,[30,31] *Bacillus,*[32,33] members of the family *Enterobacteriaceae,*[24,27,34] *Enterococcus* sp.,[35] *Lactobacillus* spp.,[36] *Pseudomonas* spp.,[24] *Streptococcus* spp.,[37,38] Non-streptococci spp.,[39] and *Candida* sp.[27,38] Present study also noted mono species biofilms of *Lactobacillus* spp. on removable appliances, worn during mixed dentition age. The species of *lactobacilli* are associated with dental caries development and also have well documented antimicrobial properties.[40,41] According to Yli-Knuuttila *et al,*[42] *Lactobacilli rhamnosus* GG could not colonise in the oral cavity while taking it as probiotics for short duration, though it reduced initial caries in kindergarten children in long term exposure.[43] Moreover, probiotic *Lactobacillus reuteri* is able to form biofilms.[44] The week negative correlation with *Enterobacteriaceae* and non-streptococci partially reiterates the previous findings as no correlation was established among oral *streptococcus* with *Lactobacillus* species. Besides this, there was a positive correlation shown between anaerobic bacteria and *Lactobacillus* species, was not supportive to the previous findings[45] and could be explained by the species variation.

CONCLUSIONS

Wide variation of microbial composition and quantity was observed during this study and was in accordance with previous investigations. Findings of our study indicate that the environmental, dietary, and genetic factors influence the individual's oral microbiome. These findings are in accordance with previous studies.[44, 45] Presence of a acrylic baseplate on the oral mucosa by itself alters the local environmental conditions due to the hampered natural cleaning mechanism of saliva and the tongue resulting into multi species biofilms on acrylic devices. Halitosis, palatal stomatitis, higher proximal caries progression, gingivitis and ultimately the loss of compliance can be the possible consequences of undisrupted biofilms on acrylic surface. Therefore, an extra hygienic measure is essential while wearing acrylic devices in oral cavity. Further study is needed to evaluate the effect of various hygiene procedures on complete disinfection of removable orthodontic appliance.

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