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Journal of Oral Infection and Pathology

RESEARCH ARTICLE

Quantitative Assessment and Comparision of Increase in *S. Mutans* with Orthodontic Treatment in Self Ligating Vs Conventional Ligating Brackets: A Real Time PCR Evaluation

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Citation: Thapa A, Kumar M, Jayan B, Chopra SS, Kumar AN, et al. (2019) Quantitative Assessment and Comparision of Increase in *S. Mutans* with Orthodontic Treatment in Self Ligating Vs Conventional Ligating Brackets: A Real Time PCR Evaluation. J Oral Infect Pathol 1: 101

Abstract

Objective: This study was taken up with null hypothesis that there is no difference in increase in Streptococcus mutans (*S. Mutans*) count before the start of treatment and twelve months after bonding in SLB (Self ligating bracket) and CLB (Conventional ligating bracket) system.

Methods: The study sample consisted of 30 patients selected from the orthodontic OPD and were divided into two groups based on the random number allocated to the selected subjects. Collection of biofilm was done from labial surface of lower teeth from selected 30 patients pre-bonding and twelve months after bonding. A portion of 100µl of each sample was used for the colony counting assay and the rest was stored at -20 °C to facilitate the conduct of real time PCR assay for colony count.

Results: Increase in Streptococcus mutans count after bonding obtained from real time PCR for SLB system was not statistically significant (T= -1.26, P= 0.227) whereas statistically significant increase in number of microorganism was seen in mean calculated values for CLB system. (T= -3.43, P= 0.004). On comparison, mean increase of Streptococcus mutans count in SLB and CLB systems after twelve months of bonding was seen to be statistically significant (T= -2.99, P= 0.009) which indicated that significant increase in number of microorganism was seen in real time PCR values obtained from CLB system when compared to group bonded with SLB system.

Limitations: Studies comparing different sex and age group in larger samples are required.

Conclusions: The quantitative increase in *S. mutans* count over twelve months of treatment in CLB group was seen to be statistically significant.

Keywords: Streptococcus Mutants; Real Time PCR; Self ligating bracket; Conventional ligating bracket; Colony Counting assay

Introduction

Bondable brackets have numerous advantages over bands because of better esthetics, ease of placement and removal, and accessibility for oral hygiene [1,2]. The diversity of devices used in orthodontic appliances can promote specific alterations in the oral environment, such as acidic pH, greater adherence of microorganisms (Streptococcus mutans), and the development of biofilm [3-6]. These alterations increase the risk of enamel decalcification [7,8]. The porous structure of the material of the brackets provides a highly favorable ecological niche for the adherence of microorganisms and the continuous development of biofilm [9-12].

Self-ligating brackets have a mechanical mobile device to close the slot, converting it into a tube. Some systems are considered passive (Damon 2, Smart Clip) and others are active (Speed, QuicKlear). Some advantages are attributed to the self-ligating bracket systems: eg, more rapid orthodontic movement and consequent reduction in treatment time for the mechanical closing of space [13-15]. However, it is not known whether adherence of microorganisms and the development of biofilm are diminished when self-ligating brackets are used, because the ligatures (metallic or elastic) necessary for maintaining the orthodontic wire in place with conventional brackets are replaced by the opening and closing mechanism (clip) of the self-ligating brackets. The alteration of

microbial adherence depends also on factors such as variations in design, size, and composition of the self-ligating and conventional esthetic brackets, methods of bonding and of tying the wire to the slot, level of oral hygiene, and age of the patient. The following study was taken up with null hypothesis that there is no difference in increase in *S. mutans* count before the start of treatment and twelve months after bonding in SLB (Self ligating bracket) and CLB (Conventional ligating bracket) system.

Materials and Methods

This multi-disciplinary study was conducted at the Division of Orthodontics and Dentofacial Orthopedics of a tertiary care dental establishment. The study populations of 30 patients, reporting during the period Jun 2012 to Aug 2012 were selected from Out Patient Department of the Institute. According to Pandis, *et al*. the sample size of 15 patients per group at $\alpha = 0.05$ yields a statistical power close to 0.8 for this kind of study [16]. All patients required fixed appliance orthodontic treatment. The study sample was divided into two groups based on the random number allocated to the selected subjects. Group 1 consisted of 15 patients who received treatment with self-ligating preadjusted bracket system. Group 2 consisted of 15 patients who received treatment with a conventional ligating preadjusted edgewise bracket system. Similar oral hygiene and appliance maintenance instructions were given to both groups. The research protocol described in this study was reviewed and approved by the Institutional Ethical Committee, Armed Force Medical College Pune. Nature and the purpose of the study were explained to all of the participants and informed written consents were obtained from each of participants at the enrolment of this study.

The requirements for inclusion in this study were subjects having permanent dentition, undergoing orthodontic treatment for the first time, class I or Class II malocclusion necessitating a fixed orthodontic appliance treatment protocol in both maxillary and mandibular arches, a minimum irregularity of 3mm in the mandibular anterior region, based on Irregularity Index as given by Robert M. Little in 1975, ability to maintain adequate oral health. Exclusion criteria for study were subjects with previous history of orthodontic treatment, patients who were pregnant, diabetic, using mouth rinses or interacting medications, including antibiotic therapy within 3 months before study, treatment requiring asymmetric extractions, impacted or unerupted permanent teeth anterior to the first molars, patients with cleft lip and palate, severe skeletal dysgnathia and ectopic canines, patients requiring adjuncts for fixed appliance therapy, involving intermaxillary or other intraoral or extraoral appliances including lip bumpers, maxillary expansion appliances or headgear.

The patients were treated under the same protocol adopted by the institution with the following archwire sequence: 0.014-inch Damon Copper Ni-Ti (Ormco), 0.014 x 0.025-inch Damon Copper Ni-Ti (Ormco), 0.018 x 0.025-inch Damon Copper Ni-Ti (Ormco).

Dental plaque samples

Collection of dental plaque samples was done with the help of sterile paper points from labial surface of lower teeth (first molar and labial surface of canine) from selected 30 patients pre bonding and one year after bonding by single operator and placed in microcentrifuge tubes containing 200µl of sterile saline. The samples were stored in a temperature controlled environment and transferred to the laboratory immediately. A portion of 100µl of each sample was used for the colony counting assay and the rest was stored at -20 °C to facilitate the conduct of real time PCR assay.

Enumeration of bacteria by colony counting

The samples as above were inoculated onto a selective medium Mitis-salivarius-bacitracin agar (MSB agar, HiMedia Ltd India). Tenfold dilution series was prepared going down to approximately four times the original sample solution and 100 µl from each dilution was spread out evenly over a quadrant of 100mm diameter MSB agar plate. Plates were incubated anaerobically at 37 °C for 72 h in a Macintosh Filde anaerobic jar with Anero Pack (HiMedia, India limited). Smaller colonies were counted and were identified by standard biochemical test. Larger colonies were not taken into account for this study. Result of the culture assays were blinded to the investigator of the real-time PCR assay.

Bacterial strains and DNA

Bacterial strains used in this study are listed in (Table 1). PCR primers Smut3368-F and Smut3481-R anneal to conserve regions of gtfB gene of *S. mutans* were used and a 114 base pair amplicon was generated from the PCR (Table 2). Both primer pairs were designed to amplify the regions that corresponds to a section of the conserved catalytic domain of GTF-I. Primers and probes were obtained from Eurogentec, Belgium (Table 3).

Strain no.	Species group in *S. mutans*	Species and strain
1	*S. mutans* ATCC25175™	*S. mutans* ATCC25175™

Table 1: Bacterial strain

Designation	Sequence	Amplicon size (bp)	Target	Tm[a](°C)
Smut3368-F	5GCCTACAGCTCAGAGATGCTATTCT-3'	114	gtfB	52.6
Smut3481-R	5-GCCATACACCACTCATGAATTGA-3'			48.4

Table 2: Oligonucleotide primers

Designation	Sequence	Target
Smut3423T	5'-FAM-TGGAAATGACGGTCGCCGTTATGAA-BHQ-1-3'	gtfB

Table 3: Oligonucleotide probe

Isolation of genomic DNA of *S. mutans* from samples

Genomic DNA was isolated and purified using a DNA isolation kit [HiMedia, India limited] as per the manufacturer's instructions.

Standard DNA isolation from *S. mutans*

Streptococcus mutans (ATCC 25175) was obtained from Microbiologics Inc (USA). The lyophilised organism was revived as per manufacturer's instructions BHI broth. Successive 10-fold dilutions in a series were done from growth obtained in BHI broth. The standard curves for each organism were plotted for each primer-probe set by using Standard Ct values obtained from the amplification of genomic DNA extracted above solutions containing 1.7 x 100 to 1.7 x 109 CFU (Colony Forming Unit) of *S. mutans* (Figure 1). Standard curve showing the log concentration of dilution of genomic DNA obtained from samples containing 1.7 x 100 to 1.7 x 109 CFU of *S. mutans* depicted in (Figure 2). The numbers of CFU were determined by plating culture dilutions on MSB agar (HiMedia, India) plates.

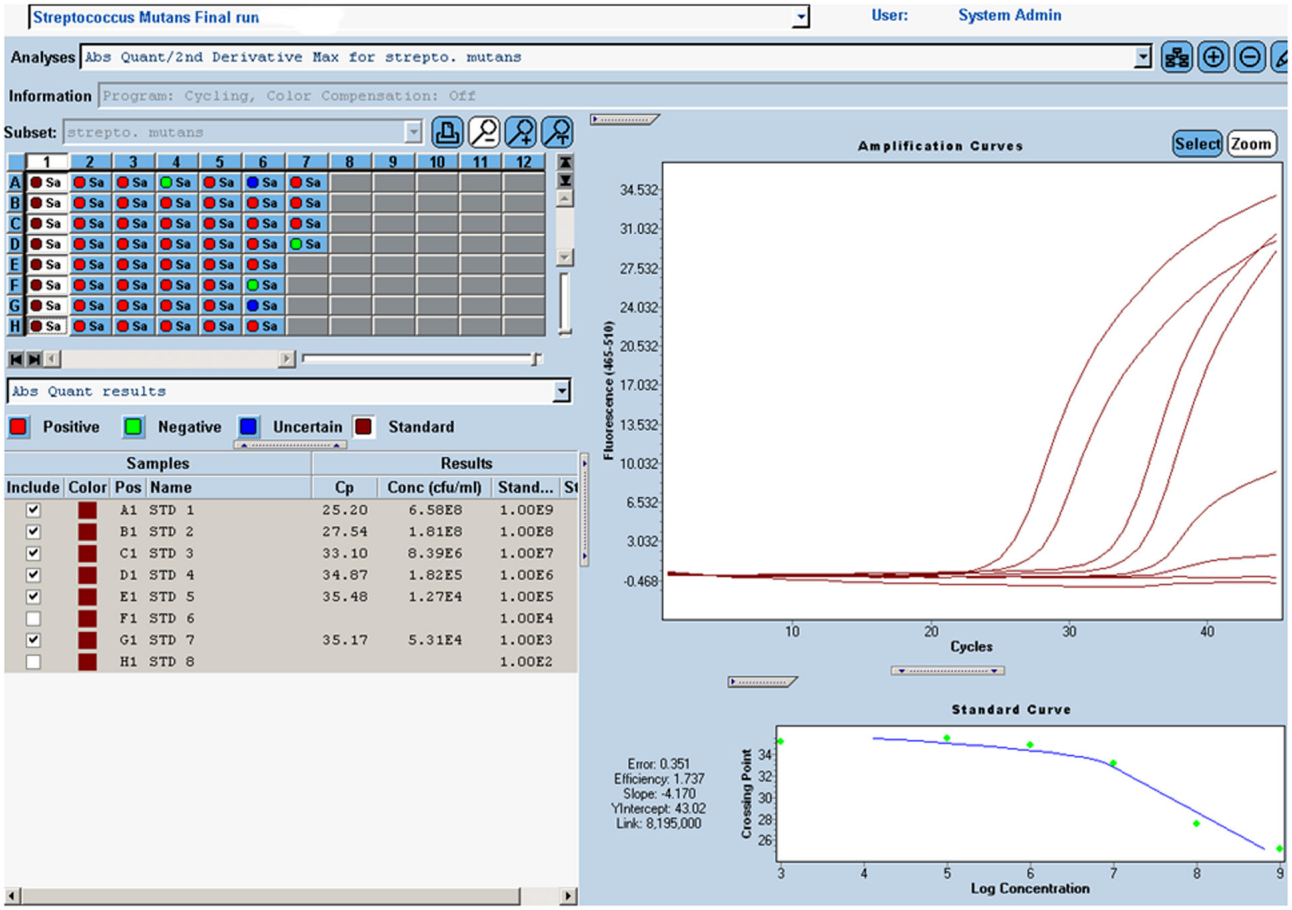


Figure 1: Standard Ct values of *S. mutans* ATCC 25175TM.

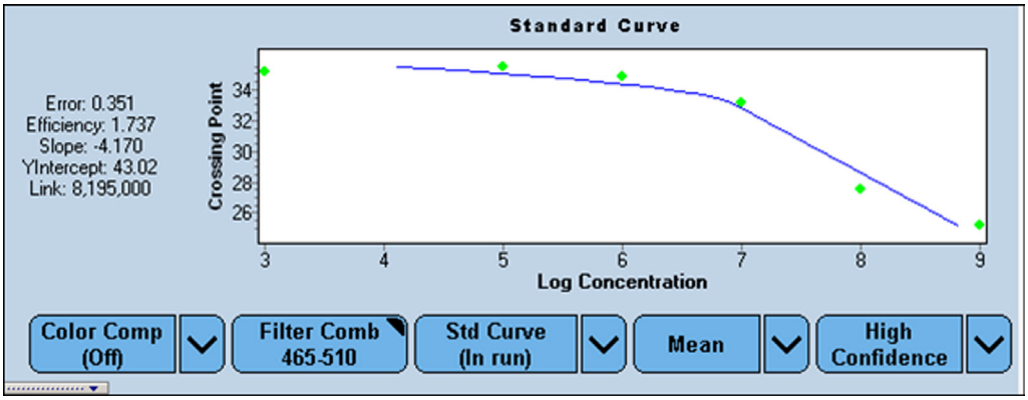


Figure 2: Standard curve of genomic DNA

Real-time PCR assay

Frozen DNA samples of patients and standard DNA which was obtained from the pure culture of *S. mutans* were thawed at 37 ºC. Real-time PCR was carried out using a cocktail containing 12.5 µl of Dream Taq PCR Master mix (Fermentas, Thermo fisher), 0.5µM each of Forward primer and Reverse primer, 250 nM Probe and 5 µl of template DNA. Total volume was made up to 25 µl with molecular grade water (HiMedia). The amplification mixtures were placed in the wells of 96-well PCR plate. DNA Amplification and detection were performed using the Roche light Cycler 480 II real time PCR machine detection system [Roche diagnostics, USA]. Thermal Cycling parameters were as follows: initial denaturation at 94 ºC for 5min, followed by 94 ºC for 30 sec and 500C for 1 min for 45 cycles. The standard curves for each organism were plotted for each primer-probe set by using Ct (Cycle of threshold) values obtained from the amplification of genomic DNA extracted from samples containing 1.7x100 to 1.7x109 CFU of *S. mutans*. Based on this approach, Ct values of clinical samples were determined (Figure 3). Ct and the CFU were correlated.

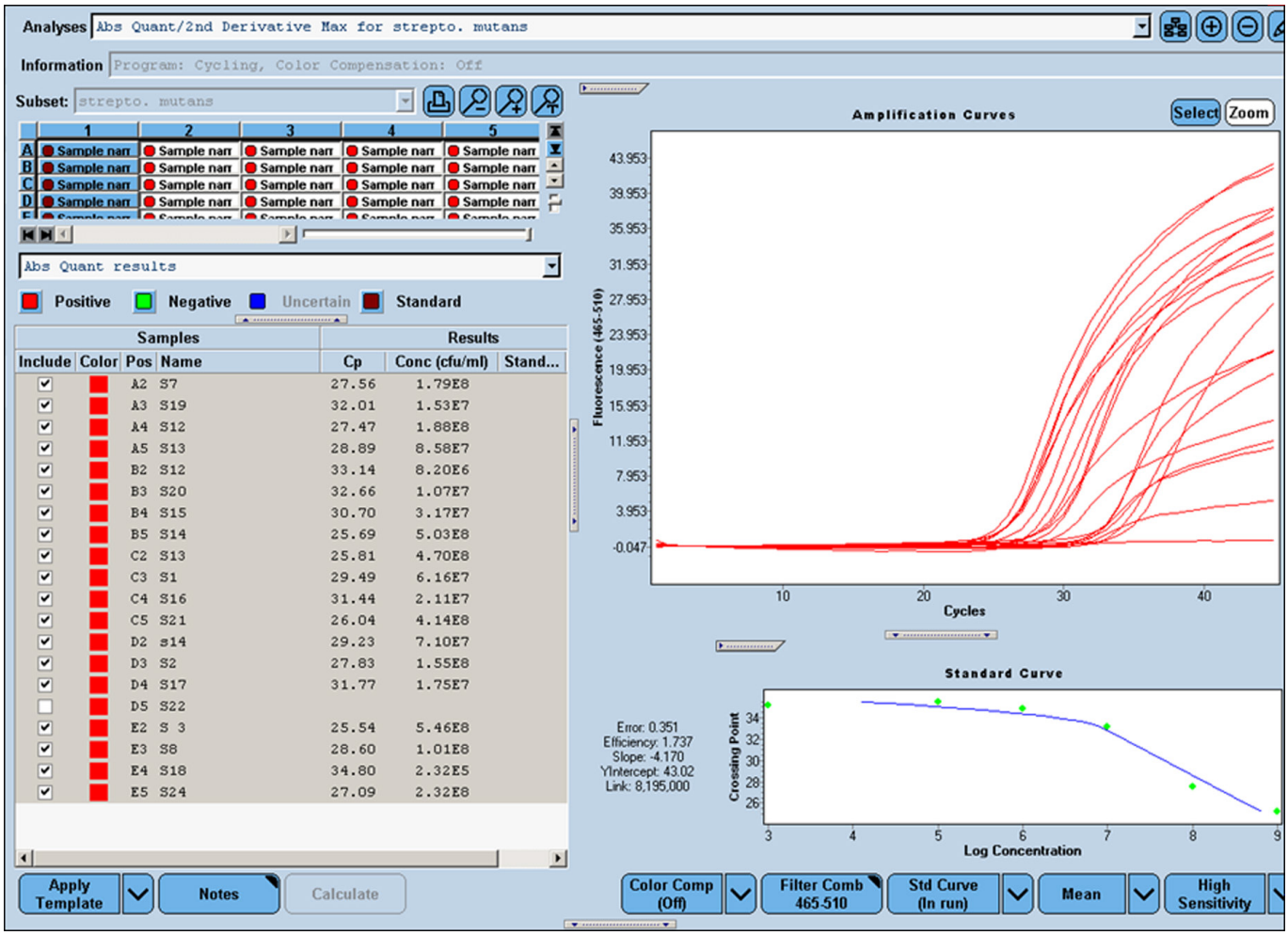


Figure 3: Ct values obtained from DNA of samples

Results

The present study was performed to compare the increase/decrease load of *S. Mutans* following a particular treatment modality. The data pertaining to all the objectives were compiled on MS excel work sheet.

Statistical Analysis

The statistical analysis of all the parameters was performed using statistical software MINITAB 1513. For all comparison within the group paired 't' test was carried out and for comparison in between two groups, samples 't' test was used. For colony count in real time PCR and Agar, log of the CFU values were taken in order to even out the variations in the individual readings. Box plot display the minimum value by end of the lower whisker and the maximum value by the end of upper whisker. The lower end of the box represents the first quartile, middle line of the box represents the median and upper edge represents the third quartile. Mean values are plotted by solid circle within the box. Asterix above and below the box represents low and high observation unusually.

Quantitative measurement of *S. Mutans* between SLB and CLB system by real time PCR

Mean calculated values obtained from real time PCR for SLB (pre-bonding) were 2497 when compared to real time PCR mean value

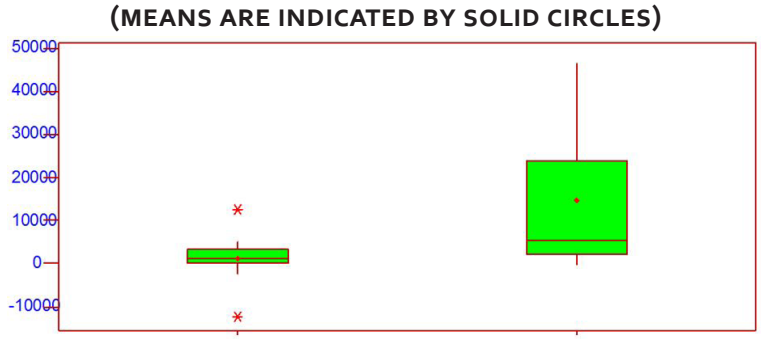
of 3983 for SLB (post-bonding). However, this increase was statistically not significant (T= -1.26, P= 0.227). This data indicates that no significant increase number of microorganism was seen in mean calculated values obtained from real time PCR for SLB after twelve months of bonding compare to pre-bonding stage.

The mean calculated values obtained from real time PCR for CLB (pre-bonding) was 1059 compared to mean real time PCR for CLB (post-bonding), which equals to 15713. The above parameters assessed had statistically significant difference (T= -3.43, P= 0.004). This data suggests that significant increase in number of microorganism was seen in mean calculated values for CLB obtained from real time PCR after twelve months of bonding compare to pre-bonding stage.

The mean increase with respect to SLB was 1295 whereas with respect to CLB equals to 14655 (Table 4). This difference between the treatments was statistically significant (T= -2.99, P= 0.009). This data indicates that significant increase in number of microorganism was as seen in real time PCR values obtained from CLB system after twelve months of bonding when compared to group bonded with SLB (Graph 1).

Real time (PCR)	N	Mean	SD	SE Mean	p Value	T Value
SLB	15	1295	5084	1313	0.009	-2.99
CLB	15	14655	16545	4272		
Difference = *S. mutans* (PCR) for SLB – *S. mutans* (PCR) for CLB Estimate for difference: -13360 95% CI for difference: (-22834, -3886) T-Test of difference = 0 (vs not =0)						

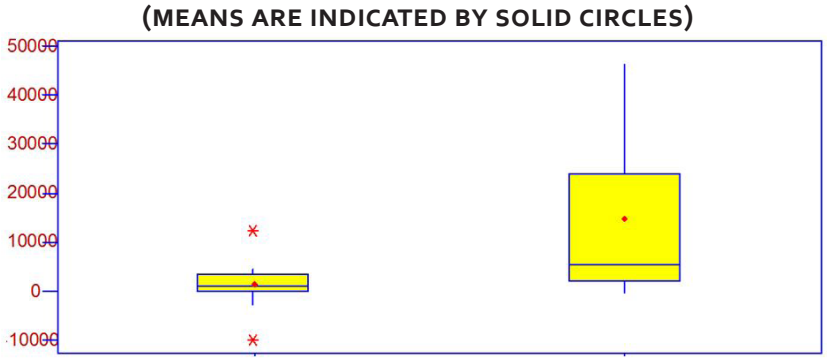
Table 4: Comparison of mean calculated values from real time PCR in SLB and CLB before and after twelve months of bonding by Two Sample T-Test (Valued scaled to 10^5)



Graph 1: Comparison of mean calculated values from real time PCR in SLB and CLB before and twelve months after bonding

Quantitative measurement of *S. Mutans* between SLB and CLB system by colony count in MSB agar

Mean of colony count (cfu/mL) in Agar SLB (pre-bonding) was 2558 while for Agar SLB (post-bonding) equal to 3985. This increase was statistically non-significant (T= -1.20, P=0.249). This data indicates that colony count (cfu/mL) in agar from plaque sample collected from SLB group before and after twelve months was not significant compared to pre-bonding stage. Mean colony count (cfu/mL) in Agar for CLB (pre-bonding) group was 1065 while mean colony count (cfu/mL) in Agar for CLB (post-bonding) equals to 15700. This increase was statistically highly significant (T= -3.43, P=0.004). This data indicates that colony count (cfu/mL) in agar from plaque sample collected from group bonded after twelve months with CLB was significantly increase compared to pre-bonding stage. The mean increase for SLB in Agar was 1426 while for CLB in Agar equals to 14635 (Table 5). This difference of colony count between Agar (SLB) and Agar (CLB) was statistically significant (T= -2.98, P= 0.009). This data indicates that colony count (cfu/mL) in agar from plaque sample collected from group before and after twelve months of bonding with CLB system was significantly increased and more when compared to SLB system (Graph 2).



Graph 2: Comparison of mean calculated values from culture method in SLB and CLB before and twelve months after bonding

Agar	N	Mean	SD	SE Mean	p Value	T Value
SLB	15	1426	4597	1187	0.009	-2.98
CLB	15	14635	16543	4272		
Difference = *S. mutans*(AGAR) for SLB – (AGAR) for CLB Estimate for difference: -13209 95% CI for difference: (-22608, -3811) T-Test of difference = 0 (vs not =0)						

Table 5: Comparison of mean colony count (cfu/mL) of *S. mutans* in SLB and CLB by using Two-Sample t-test before and after twelve months of bonding (Valued scaled to 10^5)

Correlation of colony count method by PCR and MSB agar method

Pearson correlation test for colony count by PCR and MSB agar for SLB is 0.995. This difference of colony count method by two methods for SLB is statistically significant (P=0.0001). Pearson correlation test for colony count by PCR and MSB agar for CLB is 1.000 (Table 6). This difference of colony count method by two methods for CLB is statistically significant (P=0.0001).

Colony count method		Bracket system	Pearson correlation	p-value
PCR	AGAR	SLB	0.995	0.0001
PCR	AGAR	CLB	1.000	0.0001

Table 6: Correlation of colony count method by PCR and MSB Agar

Discussion

Self-ligating brackets concept in orthodontics can be traced back several decades. Harradine reports that the concept of self-ligation is as old as that of the edgewise bracket itself and was described first by Stolzenberg in 1935 [17]. Self-ligating brackets were introduced in clinical practice to replace the existing conventional ligation methods with elastomeric and stainless steel ligatures for improving clinical efficacy [18,19].

Fixed orthodontic appliances play a negative role in oral hygiene [20]. Orthodontic bands, brackets, and archwires used during fixed orthodontic treatment impede oral hygiene procedures and cause the accumulation of microbial dental plaque by creating new retention areas [21,22]. Dental plaque is the main etiologic factor in the development of dental caries and periodontal diseases [23]. Demineralization occurs around the brackets because of a decrease in the pH level caused by increases in the number and volume of acid-producing bacteria in plaque, mainly Streptococcus mutans, Streptococcus sobrinus, and lactobacilli [24,25]. Many studies have reported increase in the amount of cariogenic microorganisms, including *S. mutans* and lactobacilli in dental plaque and saliva of patients after the bonding of orthodontic appliances [26]. During fixed orthodontic treatment pathologic changes in patients treated with fixed orthodontic appliances have been reported as mostly gingivitis, gingival bleeding, gingival enlargement and increased periodontal pocket depth. The method of ligation is an additional factor to be taken into account for microbial dental plaque retention. However, few studies have evaluated the effect of the ligation method [27]. In previous studies, although various techniques have been used for the assessment of microbial flora, the microbiologic culture technique was the most widely used [28]. However, the laboratory procedures for this technique can be faulty, time-consuming, and laborious. Recently, to overcome these limitations, polymerase chain reaction (PCR) has been used. PCR is a simple, fast, and accurate method for the identification and detection of microorganisms; in this method, specific DNA fractions are used and small numbers of pathogens can be detected in the sample [29,30]. Recently, the effects of self-ligating brackets on oral hygiene have been investigated and a few studies are available on this topic.

This present study was designed to clinically evaluate the quantitative bacterial examination of *S. Mutans* before the start of treatment and twelve months after bonding in order to reach near to the conclusion whether projected hypothesis was true or not.

Assessment of effect of two ligation system in quantitative increase in *S. Mutans*

By comparing data from the culture assay with those from the real time PCR assay, the difference in units between the CFU and the copy number of the DNA is assessed. Generally, one colony is not necessarily derived from one single bacterial cell when the cells are aggregated and rapidly growing bacterial cells usually contain more than one chromosome [31]. The numbers of copy genome DNA per CFU are influenced by the bacterial species, strain and sample condition. Thus, real-time PCR assay applicability is assessed only in terms of the contrast in data determined from both culture and real-time PCR assays on each sample. In present study, results from both assays followed almost the same trends (Figure 2).

An objective of the study was to measure the quantitative change in *S. mutans* in patients bonded with self-ligating and conventional ligating preadjusted edgewise bracket system. The results of present study compared which bracket system i.e., SLB versus CLB bracket were associated with increased growth of *S. mutans* after twelve months. To assess the quantitative increase of *S. mutans*, real-time PCR and enumeration of bacteria by colony count using MSB agar was done before and after twelve months

of bonding. In general, real-time PCR possesses several advantages over culture assays. The real-time PCR can easily reveal the individual quantities of *S. mutans* independently. A contrast culture assay needs many steps for separately quantifying *S. Mutans.* Microorganisms have to be alive and culturable for detection by plate counting. Sample conditions before plating directly affect the number of colonies. In contrast, PCR detects the DNA of microorganisms whether they are alive or not [32]. This difference influences the way in which samples are transported and stored. Samples remain stable over long periods of freezing for PCR assays. In the present study both PCR and MSB agar was used for quantitative assessment of *S. mutans* and the results indicated both the method are significantly effective for quantitative assessment (Table 6). Intra-group comparison between SLB and CLB system for assessment of quantitative increase of *S. mutans* was done; results showed no significant increase after twelve months of bonding in SLB but significant increase in number of *S. mutans* was observed in CLB system (Table 6). Inter-group comparison between SLB and CLB system was done which showed significant increase in number of *S. mutans* in patients with CLB system compared to SLB after twelve months of bonding (Table 5).

Some studies reported a negative influence of fixed orthodontic appliances on quantitative and qualitative distribution of oral microbiota, an unequivocal and consistent conclusion regarding changes of oral microbiota during orthodontic treatment is still lacking [33]. In order to obtain a more uniform conclusion regarding the influence of fixed orthodontic appliances on oral microbiota, it is necessary to conduct clinical studies for a longer duration. Jordan and LeBlanc reported similar results in their four month longitudinal study [34]. They linked the inconsistent pattern of *S. mutans* prevalence observed among their patients to the oral hygiene practices of individual patients. Hence, in the present study collection of the plaque sample was done before and after twelve months after bonding. Concurrent to our results, an in vivo study was done by Pellegrini, *et al.* suggested a higher retention of plaque bacteria on conventional ligating brackets than on SLBs [35,36]. Contrary to the findings of the present study, Baka Z M and co-authors had shown the effects of self-ligating brackets and conventional brackets with stainless steel ligatures on dental plaque retention and microbial flora [37]. They reported that numbers of *S. mutans, S. sobrinus, L. casei and L. acidophilus* were not statistically different between self-ligating brackets and conventional brackets ligated with stainless steel ligatures. Recent study from Pithon, *et al.* showed greater bacterial accumulation on SLBs than on CLB with elastic ligature [38]. In present study, steel ligatures were used instead of elastomeric rings because an elastomeric ring has been clearly shown to cause more plaque accumulation in study done by Garcez *et al.* [39].

Conclusion

There was statistically significant difference in colony count of *S. Mutans* in patients bonded with self-ligating preadjusted edgewise brackets compared to conventional ligating preadjusted edgewise bracket system. The quantitative increase was significantly high for patients treated with conventional ligating preadjusted edgewise bracket system compared to self-ligating preadjusted brackets.

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