Correlation between Streptococci Mutans and salivary IgA in relation to some oral parameters in saliva of children

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ABSTRACT

Background: Saliva plays an important role in oral health. Several salivary proteins are involved in the antimicrobial defence mechanism and are able to eliminate or inhibit bacterial growth in the oral cavity. Secretory IgA (SIgA) is one of the principal antibodies present in saliva, could help oral immunity by preventing microbial adherence, neutralizing enzymes and toxins.

The aim of this study was to investigate the relationship between salivary Streptococcus Mutans (SM) count and S IgA in stimulated whole saliva in children with primary dentition compared to those with permanent teeth in relation to some oral hygiene parameters.

Material and methods: Stimulated whole saliva was collected from 50 children (25 with primary dentation and 25 with permanent teeth). Salivary flow rate and pH was measured. Oral hygiene index (OHI) and gingival inflammation was assessed using classical method. S IgA level was measured using an immunoassay kit and SM count was determined by culture media.

Results: Mean salivary flow rate, pH and S IgA were significantly higher among children with permanent teeth compared to those with primary dentition.

Compared to primary dentition, permanent dentition was associated with a significantly reduced mean plaque index, increased mean gingival index and reduced mean salivary SM count.

Although plaque index showed a statistically significant positive correlation with SM count, gingival index showed a weak negative correlation with bacterial count.

S IgA, PH and flow rate showed a statistically significant moderately strong negative correlation with bacterial count.

Conclusion: This study showed a clear correlation between count of SM in stimulated whole saliva and both SIgA and plaque index.

Keywords: Mutants Streptococci, salivary IgA, salivary flow rate. **(J Bagh Coll Dentistry 2014; 26(1):71-79).**

INTRODUCTION

Whole saliva as an external biological fluid, is frequently used an easily accessible and obtainable secretion with an interesting scientific and clinical potential. Stimulated salivary secretion may be preferable as a test sample [(1-3)]; it is more easily collected and less adversely affected by storage than unstimulated salivary secretion [(4)].

Whole Saliva contains several antimicrobial components that mediate selective adhesion and colonization of SM on the tooth surfaces. Agglutinins include mucins, glycoproteins, fibronectin, lysozyme and salivary Immunoglobulin A (SIgA) promote agglutination of SM and enhance bacteria removal. This may inhibit SM adherence to saliva-coated hydroxyapatite and epithelial surfaces and neutralize SM enzymes and virulence factors [(5-7)].

SIgA is one of the principal antibodies present in saliva which predominates in most external secretions [(8)].

SIgA in whole saliva is the contributions from the minor and various major glands vary greatly according to the flow rate [(9)].

SIgA is produced by local plasma cells in the stroma of salivary glands and is transported through secretory epithelia by the polymeric Ig receptor (membrane secretory component) [(10)]. At least 95% of the IgA normally appearing in saliva is produced by local plasma cells in the various salivary glands and transported into salivary fluids as SIgA dimers or larger polymers [(10)]. It is the first line of host defence against pathogens which invade mucosal surfaces. SIgA antibodies could help oral immunity by preventing microbial adherence, neutralizing enzymes, toxins, and viruses; or by acting in synergy with other factors such as lysozyme and lactoferrin [(11-13)].

Local immunity and antibodies may be of prime importance in defence mechanisms against infection to oral mucosa. Interaction between oral microbial flora and host response may affect the periodontal health status; periodontal disease is assumed to be associated with immunological reactions against the action of microorganisms in dental plaque [(14)]. The concentration of SIgA is directly and positively correlated with the severity of periodontal inflammation [(15)].

Research on plaque formation has shown that children with primary dentition, after professional tooth cleaning, form less plaque than older subjects over different periods of observation [(16-18)]. This demonstrates that plaque formation rate is

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low and may have the amount of established plaque over time [19]. Bacterial profiles change with disease states and differ between primary and secondary dentitions [20].

Children with primary dentition respond to plaque accumulation with less gingivitis than adults [16-18]. In children, immunological system may be acting in the presence of gingival inflammation since periodontal disease is assumed to start in childhood in the form of gingivitis, reaching a peak close to puberty and then progressing to the typical and overt of periodontitis in adults. Thus it would be of great interest to investigate the immunological defence mechanism during childhood with the advantage of longer survival time of the teeth and improving of general health condition physically and psychologically [21].

MATERIAL AND METHODS

1. Study population
The study was conducted in Al-Ramadi city in the western division of Iraq. The sample comprised of 50 children divided into two groups according to their dentition. The first group consist of 25 children with primary (deciduous) dentition aged 6-7 years and had been selected randomly from Dome of the Rock primary school in Al-Ramadi city. The second group of 25 children were with only permanent dentition, aged 12-years. In this study, all children were with no history of systemic disease and did not take any antimicrobial agents during the last two weeks prior to the study. Children who had suffered from upper respiratory tract infection in the past one week were excluded from the study due to development of IgA and lysozyme. All parents/guardians signed consent forms to allow their children to enrol into this study.

2. Collection and processing of salivary samples
Under standard temperature and humidity conditions, children were comfortably seated and, after a few minutes of relaxation, they were trained to avoid swallowing saliva, stimulated whole saliva using a piece of Arabic gum was collected from each child by expectoration into a sterile graduated test tube. All saliva samples were collected in the morning between 8.30 to 9.30 a m for 5 minutes, then the salivary flow rate was measured as millilitre per minute (ml/min)[22].

The salivary pH was measured immediately using electronic pH meter then samples were put in ice container until transport to the laboratory.
Before collection of salivary sample, oral hygiene status of the children was determined by recording the oral hygiene index (OHI) using the classical

methodology [23]. Following salivary sampling, the gingival inflammation was assessed by using the criteria of gingival index system [24].
In the laboratory of Baghdad teaching hospital, the biochemical analysis of salivary samples has been done. Before analysis, salivary samples were divided into two portions; one for salivary IgA estimation and the second was for SM counting.

3. Counting of salivary Mutants Streptococci
The viable count of bacteriaafter serial 10-fold dilutions of salivary sampleswas monitored using sterile normal saline, and immediately agitated for 30 seconds on a vortex mixer. Using adjustable micropipette with disposable tips,0.1 ml of the dilution was taken and then spread in duplicate using a sterile microbiological glass spreader on the plates of Mitis Salivarius Bacitracin Agar (MSBA);a selective media for MS.Then the agar plates were incubated both anaerobically in an anaerobic jar for 48 hours and aerobically for 24 hours at 37°C.

Identification of MS includes:
a) Colony morphology: the colony on MSB agar was examined directly and under dissecting microscope (magnification ×15); it appears as a light blue in colourwith 1-2mm in diameter.
b) Morphology of the microbial cells: a colony was picked up from MSB agar separately under sterilized condition and subjected to gram`s stain.MS cellsare gram positive, spherical or ovoid in shapewith raised or convex surface arranged in short or medium length.
c) Biochemical tests: Bacterial colonies were picked up from MSB agar under sterilized conditions using inoculating loop, inoculated in 10 ml of sterilized Brain Heart Infusion Broth (BHI-B) and incubated aerobically at 37°C for 18 hrs. Then the following tests were conducted:
➤ Catalase Production test: Hydrogen peroxide 3% (H_2O_2) had been used to detect the activity of catalase enzyme production.
➤ Carbohydrate fermentation test: mannitol media was used to test the ability ofMS to ferment the mannitol which was added in a concentration of 1% to the CystineTrypticase Agar (CTA) - mannitol media.

In biochemical tests, all colonies of SM were catalase negative and had the ability to ferment mannitol. A positive reaction is indicated by changing in the indicator colour from red to yellow by the formation of acid.

After identification, microbial counts of MS were recorded by colony counter taking in consideration the dilution factor and expressed as colony forming unit multiplied by the dilution factor per millilitre saliva (CFU/ml).

4. Estimation of total secretory salivary IgA

Salivary samples were centrifuged at 1000 x g (xg: is a relative centrifugal force) for 15 minutes; the clear supernatant was aspirated by disposable micropipette and was then frozen at -20°C until thawed for the antibody assay[25].

Salivary immunoglobulin was determined using Radial Immuno diffusion plate (RID), immunoglobulin concentration was expressed in mg/dl [26].

This technique involves immuno precipitation in agarose, between an antigen and its homologous antibody. It was performed by incorporating one of two immune reactants (usually antibody) uniformly throughout a layer of agarose gel, and then the other reactant (antigen) was introduced into the wells punched in the gel. Antigen diffused radially out of the well into the surrounding gel-antibody mixture, and the visible ring of precipitation was formed where the antigen and antibody had reacted. A quantitative relationship dose exists between ring diameter and antigen concentration, while the precipitate is explaining, the ratio between ring diameter and antigen concentration logarithm is approximately linear.

The procedure involved:

➤ The plate was opened and left for five minutes at room temperature to allow any possible condensation to evaporate.

➤ The well was filled with 5μl of saliva and the plate was closed tightly.

➤ The plate was allowed to stay flat at room temperature until the precipitation ring reach its maximum possible size, which often required 48-72 hours of diffusion with the end point ofdiffusion was identified by sharp precipitation ring at the end of the incubation time. The diameter of the ring was measured by a lens and then the results were calculated using the reference values.

Statistical Analysis

Statistical analyses were done using SPSS version 19 computer software (Statistical Package for Social Sciences). Statistical significance of differences in mean for normally distributed parameter between two groups was assessed using the Student's t-test. P value less than 0.05 was considered statistically significant.

The statistical significance, direction and strength of linear correlation between two quantitative normally distributed variables were measured by Pearson's linear correlation coefficient.

A multiple linear regression model was used to study the net and independent effect of a set of explanatory variable on a quantitative outcome (dependent) variable. The model provides the following parameters:

P-value (model): In order to generalize the results obtained, the model should be statistically significant.

Unstandardized partial regression coefficient: Measures the amount of change expected in the dependent variable for each unit increase in the independent variable after adjusting for other explanatory variables included in the model. Standardized regression coefficient: Measures the relative importance of each independent variable.

P-value for regression coefficient: Reflects the statistical significance of the calculated partial regression coefficient of each explanatory variable included in the model.

R squared (R^2) (Determination coefficient): Measures the overall performance of the model since it reflects the amount of variation in the dependent variable explained by the model. The closer its value to 100% the better the model fit.

Cohen's d is an effect size test used to indicate the standardised difference between two means. It can be used for t-test and would be an appropriate accompaniment to inferential testing.

RESULTS

The results presented in this study were based on the analysis of a random sample of 25 children with permanent dentition (age =12 years) and 25 children with primary dentition (age =6-7 years). Gender distribution was equal in the two groups.

1. Salivary parameters

As shown in table 1, the mean salivary flow rate was significantly higher among cases with permanent dentition (0.83 ml/min) compared to those with primary dentition (0.7 ml/min). Permanent dentition is associated with a mean increase in salivary flow rate of 0.13 compared to the primary dentition with Cohen's d was 0.93 which reflect an effect size used to indicate the standardized difference between two means.

The mean salivary pH was significantly higher among cases with permanent teeth (7.3) compared to those with primary dentition (6.6). Permanent dentition is associated with a mean increase in salivary pH of 0.7 compared to the primary dentition with Cohen's d. was 1.72.

The mean salivary IgA was significantly higher among cases with permanent teeth (21.2) compared to those with primary teeth (18.2). Permanent dentition is associated with a mean increase in salivary IgA of 3 compared to the primary dentition.

The effect of permanent dentition on the previously mentioned indices compared to those

with primary dentition were all evaluated as high effect, since its Cohen's d was higher than 0.8.

The effect of permanent teeth was higher on salivary pH followed by salivary IgA (Cohen's d=1.72 and 1.62 respectively), while it was lowest on salivary flow rate (Cohen's d=0.93).

2. Oral health status

As can be seen in table 2, the mean plaque index was significantly lower among children with permanent teeth (1.4) compared to those with primary dentition (1.7). Permanent dentition is associated with a mean reduction in plaque index of 0.3 compared to primary dentition. This effect was a strong one (Cohen's d =-1.33).

The mean gingival index was significantly higher among cases with permanent teeth (1.7) compared to those with primary teeth (1.5). Permanent dentition is associated with a mean increase in gingival index of 0.2 compared to the primary dentition. This effect was a moderately strong effect (Cohen's d = 0.7).

3. Mutans Streptococci count in whole saliva

The mean salivary count of MS was significantly lower among cases with permanent teeth (5.6 ± 4.1) compared to those with primary teeth (14 ± 4.44). Permanent dentition is associated with a mean reduction in salivary bacterial count of 8.4x100 compared to the primary dentition. This effect was a strong one (Cohen's d =1.97), figure 1.

A linear correlation coefficient was calculated between the MS count and selected quantitative variables such as salivary IgA, salivary pH and salivary flow rate, showed a statistically significant moderately strong negative (inverse) linear correlation with bacterial count (r=-0.46,-0.40 and -0.40 respectively $p < 0.05$).

Plaque index showed a statistically significant moderately strong positive linear correlation with bacterial count (r=0.43, $p < 0.05$). Gingival index on the other hand had a weak and statistically non-significant negative linear correlation with bacterial count (r=-0.12, p=0.42).

To explain the reduction observed in count of cariogenic bacteria (MS) in cases with permanent dentition as compared to children with primary dentition, a multiple linear regression modeling was performed in the overall sample (n=50) with MS count was used as the dependent (response) variable.

Two models were used to account for the confounding effect of salivary flow rate on salivary IgA.

In the first model, three explanatory variables were adjusted for (Salivary pH, plaque index and gingival index to show the net and independent protective effect of salivary IgA on bacterial count. The model was statistically significant and able to explain 34% of variation in the response variable (bacterial count). After adjusting for the possible confounding effect of salivary pH, plaque index and gingival index, it was found that for each unit increase in salivary IgA the bacterial count is expected to significantly decrease by 0.77 (x100). For each unit increase in salivary pH the bacterial count is expected to decrease by 1.6 (x100) after adjusting for the remaining explanatory (independent) variables included in the model. This effect was however not significant statistically. For each unit increase in plaque index the bacterial count is expected to significantly increase by 7.1 (x100) after adjusting for the remaining explanatory (independent) variables included in the model, table 3.

In the same way for each unit increase in gingival index the bacterial count is expected to increase by 0.6 (x100) after adjusting for the remaining explanatory (independent) variables included in the model. This effect was however statistically non- significant. In this model plaque index had the strongest effect on bacterial count followed by salivary IgA (as shown by the high value of standardized coefficients). Salivary PH and gingival index had a much less important effect in deciding the observed MS count (p-value was non-significant for both; table 3).

In the second model, salivary flow rate was introduced as the fifth independent variable to model-I. The resulting model was also statistically significant and able to explain 40% of the observed variation in the dependent variable (bacterial count). Salivary flow rate took over the place of salivary IgA in the model. It ranked second in its importance after plaque index (as shown by its standardized coefficient). For each unit increase in salivary flow rate the bacterial count is expected to significantly decrease by 12.7 (x100) after adjusting for the remaining explanatory variables included in the model. The role of salivary IgA in deciding the bacterial count ranked third now and it lost its statistical significance. For each unit increase in salivary IgA the bacterial count is expected to decrease by 0.37 (x100) after adjusting for the remaining explanatory variables included in the model, table 4.

DISCUSSION

In this study, investigation of salivary flow rate, pH, SIgA and MS count in the stimulated whole saliva, beside some oral health status parameters were performed in a group of children having primary teeth and a group with permanent teeth for assessment, evaluation and comparison purposes.

The present study showed that the mean salivary flow rate and pH was significantly higher among children with permanent teeth compared to those with primary dentition. This finding agrees with previous studies showed an increase in whole salivary flow rate with age [27-29]. This may be attributed to the process of salivary gland maturation associated with age growing of child which may favour the higher values of salivary pH found in this group of children.

The current study showed that permanent dentition is associated with reduction in the mean plaque index and higher mean gingival index compared to primary dentition. While primary dentition exhibited higher plaque index and lower gingival index compared to permanent teeth. According to Mackler and Crawford, the most acceptable explanation for the absence of clinical gingivitis in cases is the innate resistance and presence or absence of host response in this age group [30].

Permanent dentition is associated with lower salivary SM count compared to primary dentition. In this study, there is no relation between gingival inflammation, amount of plaque and SM count in the whole saliva of both groups of children. This is consistent with a study that investigated the correlation between salivary SM counts and both of plaque amount and gingival inflammation in school children; which found that there was no association between counts' of SM in saliva with plaque amount and gingival inflammation [31].

The higher values of both plaque index and salivary SM in children with primary teeth in the present study may be due to lack of good personal practices of oral hygiene in this age group of children. Oral hygiene in lower age children with primary teeth, by the time they should be more responsible for maintaining good oral hygiene status [32]. This group of children compared to those with permanent teeth have different dietary habits include frequent sugar intake, fruit juices, sweet solids and drinks favours the growth of bacteria such MS [33]. The high percentage of salivary MS in children with primary teeth in the current study may be related to the lower salivary flow rate and pH [34] in those children, affecting the accumulation and maturation of biofilm at the gingival margin [29]. This may support the results of this study of lower salivary flow rate and pH in relation to high MS count.

Secretory IgA is the main immunoglobulin in salivary fluid. Data provided evidence that children have salivary IgA antibodies shortly after birth, which might influence the establishment of the oral microbiota [35]. Biologically, salivary SIgA provides the first line of immune defence in the oral environment, responsible for inhibiting the bacterial adhesion on the enamel and epithelial cells and may be acting in synergy with other defence mechanisms to inactive bacterial enzymes/toxins and activating the complement; it is partially involved in cell-mediated immune responses [36-37].

In the present study, permanent dentition is associated with an increase level of SIgA compared to primary dentition. This is consistent with the view of increasedsalivary IgA concentrations with age which has been reported by previous study [38] and might reflect a developing immune response in the growing child [39]. According to Thaweboon et al., SIgA has a parabolic relationship with age; at birth SIgA levels are undetectable but with age there is a consistent increase in the levels [40]. This finding supports the current study finding which emphasizes the relationship between age and SIgA concentration.

Previous studies have demonstrated that salivary IgA, pH and flow rate play an important role in the oral mucosal defence mechanisms [41 and 42]. This study showed an increased level of both flow rate and S IgA concentration in children with permanent teeth. However, this is inconsistent with the findings of Kugler et al (1992) who demonstrated a significant inverse relationship between salivary flow rate and salivary IgA concentration [38].

SIgA may inhibit the attachment of oral streptococci to teeth and can enhance lactoferrin, peroxidases and lysozyme activities in saliva, subsequently reducing SM colonization [43]. This may confirm the findings of this study which showed a strong negative correlation between SM count and SIgA level.

For the purpose of confirmation and more explanation of the current study findings, multiple linear regression analysis was performed using overall study samples; with SM count considered as dependant variable. In the first model, adjustment for the salivary pH, plaque and gingival index was generated to study the effect of SIgA on SM count. Regression analysis showed that the most significant and strongest effect on SM count was for plaque index followed by SIgA level. For each unite increase in plaque index the SM count is expected to increase 7-time (7.1 x100), whilst a significant reduction in SM count to 77% (0.77 x100) was seen when there is a unite increase in SIgA concentration.

The study finding of significantly positive correlation between plaque index and SM count is confirmed by above interpretation, which is also supported by other studies which found that SIgA may help maintain disease-free oral cavity by limiting microbial adherence to epithelial

tissue/tooth surfaces via neutralizing virulence factors and also by preventing the penetration of antigens into the oral mucosa [40]. Furthermore, higher levels of microbial antigenic loads present in the oral cavity of these children probably increases the immune reaction which leads to high levels of antibody production [40].

In the second model, salivary flow rate was introduced as an independent variable and ranked second after plaque index to influence the bacterial count. For each unite increase in salivary flow rate, SM count is expected to increase by around 13 time (12.7 x100).

There are many factors that can influence the concentration of SIgA[38]. Salivary flow rate is considered as an important factor in determining the concentration of SIgA[44] and it is controlled by several factors, such as food ingestion, sensory stimulation, drugs, smoking, body positioning, stress and degree of hydration. Dietary factors, daily mood, and intense physical activity may also affect SIgA concentration [45,46].

It is worth noting that comparison of all results with other studies was not possible. This may be due to disparity between results which could be attributed to either differences in study population related to dietary pattern, oral hygiene practice and genetic factors or to the technique of saliva collection and laboratorial tests used.

In conclusion this study found that:
- Children with permanent dentition exhibited a higher salivary flow rate, pH and SIgA levels compared to those with primary teeth.
- Children with permanent dentition showed lower plaque index and higher gingival index compared to those with primary teeth.
- SM count was lower in cases with permanent teeth compared to those with primary teeth.
- A negative correlation was found between SM count and each of flow rate, pH and SIgA levels.
- Plaque index and SM count is positively correlated, and has the strongest effect on bacterial count.
- Salivary flow rate comes after plaque index to influence the SM count.

Acknowledgments

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J Bagh College Dentistry   *Vol. 26(1), March 2014*   *Correlation between*

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Oral Diagnosis   77

Table 1: Mean differences, range and size effect (Cohen's d) of selected explanatory variables.

	Children with primary dentition (age =6-7; N=25)	Children with permanent dentition (age =12; N=25)	P value (t-test)
1. Salivary flow rate			0.003
Range	(0.59 - 0.83)	(0.68 - 1.46)	
Mean	0.7	0.83	
SD	0.061	0.189	
SE	0.012	0.038	
Difference in mean	0.13		
Cohen's d	0.93		
2. Salivary PH			<0.001
Range	(5.5 - 7.3)	(7 - 7.7)	
Mean	6.6	7.3	
SD	0.53	0.22	
SE	0.11	0.04	
Difference in mean	0.7		
Cohen's d	1.72		
3. Salivary IgA			<0.001
Range	(15.7 - 21)	(16.7 - 25.1)	
Mean	18.2	21.2	
SD	1.6	2.08	
SE	0.32	0.42	
Difference in mean	3		
Cohen's d	1.62		

Table 2: The difference in the mean of two secondary outcome variables; plaque and gingival index.

	Primary dentition	Permanent dentition	P-value (t-test)
1. Plaque index			<0.001
Range	(1.4 - 2.2)	(1 - 2)	
Mean	1.7	1.4	
SD	0.2	0.25	
SE	0.04	0.05	
Difference in mean	0.3		
Cohen's d	-1.33		
2. Gingival index			
Range	(0.8 - 1.8)	(1.2 - 2.3)	0.01
Mean	1.5	1.7	
SD	0.3	0.27	
SE	0.06	0.05	
Difference in mean	0.2		
Cohen's d	0.7		

Table 3: Multiple linear regression model with MS bacterial count (x 100) as the dependent variable

|	Model-I Partial regression coefficient		P-value
	Unstandardized	Standardized	
(Constant)	24.3		0.05 [NS]
Salivary PH	-1.6	-0.141	0.36 [NS]
Plaque index	7.1	0.328	0.014
Gingival index	0.6	0.031	0.81 [NS]
Salivary IgA	-0.77	-0.307	0.045

NS=Non-significant, R_2=0.34
P-value (Mode l) <0.001

Table 4: Multiple linear regression model with MS bacterial count (x 100) as the dependent variable.

	Model-II Partial regression coefficient		P-value
	Unstandardized	Standardized	
(Constant)	20.5		0.09 [NS]
Salivary PH	-1.3	-0.115	0.44 [NS]
Plaque index	8.8	0.402	0.003
Gingival index	1.3	0.066	0.59 [NS]
Salivary IgA	-0.37	-0.146	0.36 [NS]
Salivary flow rate	-12.7	-0.325	0.024

$R_2=0.40$
P-value (Mode II) <0.001
NS=Non-significant
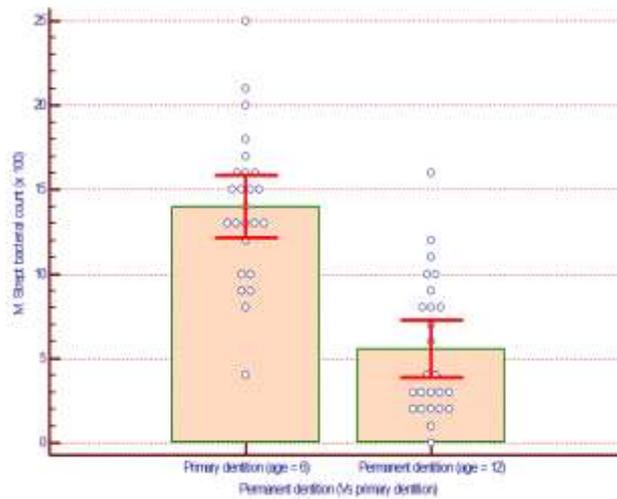
Figure 1: Dot diagram with error bars showing the difference in mean (with its 95% confidence interval) primary outcome variable (salivary MS bacterial count).
Difference in mean = 8.4
Cohen's d = -1.97