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ORIGINAL ARTICLE

A CORRELATION STUDY OF TOTAL SALIVARY COUNTS AND VIRULENT MARKERS OF *STREPTOCOCCUS MUTANS* WITH CARIES EXPERIENCE

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Abstract:

The aim of the study was to evaluate an impending relationship between salivary Streptococcus mutans count, sucrose dependent glass adherence, and water insoluble glucan synthesis of isolated strains of Streptococcus mutans with caries experience among young adults between the age group of 20 to 23 years which may enable future planning of caries prevention in adults. A total of 70 dental students undergoing compulsory rotary internship were selected for the study based on certain inclusion and exclusion criteria. After assessing DMFT and DMFS of the subjects, stimulated saliva was collected for the above mentioned analysis. Statistical analysis was done using student t test for comparing the means of the results of the two groups. Before applying t test the variances of both the groups was tested using Levene's test. Dental caries experience was correlated with sucrose dependent adherence insoluble glucan production and salivary Streptococcus mutans count using Pearson Correlation. Out of 70 adults only 10 were caries free, with DMFT = 0 and 60 adults presented with caries experience who had DMFT of 3 to 8 with the mean value of 5.58 and DMFS of 13 to 24 with the mean value of 17.3. A strong correlation was found between DMFS and water insoluble glucan synthesis and a positive correlation was also found between DMFS and salivary Streptococcus mutans count. There was a highly significant correlation between DMFT and adherence to glass and also water insoluble glucan synthesis,

KEYWORDS:

DMFT, DMFS, *Streptococcus mutans*, virulence test, Caries correlation.

INTRODUCTION

High quality oral health care is crucial for long term overall health which is a major resource for social, economic and personal development of the individual as well as society. Dental caries is the most common disease in the world today with very high morbidity potential. Dental caries is a progressive irreversible transmissible bacterial disease that results in demineralization of the inorganic constituents and dissolution of the organic substance of the tooth thereby leading to cavity formation and loss of tooth. A number of studies have shown high level of *Streptococcus mutans* in caries active individuals than in caries free individuals and also increase in salivary *Streptococcus mutans* counts have been observed with onset and progression of caries. Cariogenic potential of *Streptococcus mutans* is due to its ability to adhere to the initial acquired film, synthesize soluble and insoluble glucan. Synthesis of insoluble glucan from sucrose mediated by cell associated Glucosyl transferase (CA-GTF) has been found to be major virulent factor

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(Klein *et al*., 2009). A variety of predictors have been examined with caries increments including past caries experience, salivary buffering capacity, salivary Streptococcus mutans counts and GTFs. Mostly total salivary *Streptococcus mutans* counts have been taken as reliable indicator for predicting caries risk for any individual. *Streptococcus mutans* count alone is not sufficient to give a reliable indication of caries risk for an individual (Kohler and Krasse ,1990) and the ability to synthesize insoluble glucan mediated by GTFB and GTFC (Cell associated GTF) has been proposed as a marker for dental caries activity (Vaccca-Smith et al., 2007). The ability to synthesize WIG can be determined by estimation of water insoluble glucan produced in the presence of sucrose. Sucrose dependent adherence to glass occurs through water insoluble glucan (WIG) synthesized by CA-GTF in the presence of sucrose. The study included a known virulent property directly involved in the pathogenesis of caries and it was assumed that WIG synthesis is a potential marker for assessing caries activity and the present study was carried out to detect the association between independent variables i.e total salivary mutans count, sucrose dependent glass adherence property, WIG synthesis, and dependent variables DMFT/DMFS so that a reliable indicator can be used to predict and monitor caries risk including higher caries risk individuals.

MATERIALS AND METHODS

The study was approved by human ethical committee of Sri Ramakrishna dental college Coimbatore and informed consent was obtained from all the participants. The present study was carried out with dental students undergoing compulsory rotary internship of Sri Ramakrishna Dental College of age group 20 to 23. A total of 70 subjects were selected based on inclusion and exclusion criteria.. Those who had debilitating systemic diseases , history of fluoride use, antibiotic for the last 2 month, on orthodontic appliances ,and who had undergone professional measures to remove plaque and calculus in the past 15 days were excluded from the study.

EVALUATION OF CARIES STATUS

Before starting the study evaluation of the caries status was done for the willing participants in the study. The examination was done with the aid of a dental mirror and explorer after the teeth had been dried with compressed air by a single calibrated examiner. DMFT (Decayed, missing and filled teeth) and DMFS (Decayed, missing and filled surfaces) were recorded as per WHO criteria (1997). Subjects were divided into caries and caries free groups. 85.7% had caries and 14.3% were free from caries

Group A: Caries subjects with DMFT ranging from 3 to 8 and DMFS ranging from 13 to 24 (60 subjects)
Group B: Caries free subjects with DMFT and DMFS 0 (10subjects)

Enumeration of salivary *Streptococcus mutans* count of the participants

Participants were asked to refrain from eating for one hour before saliva collection. Two samples of saliva were collected at an interval of 4 days. After chewing with sterile paraffin wax for 3 minutes 2 ml of induced saliva was collected from the subjects in a calibrated sterile container, identified by a code number during the period of sample collection and processing. Samples were transported in ice box within half an hour to the laboratory for immediate processing. Saliva was vortex mixed in a cyclomixer for 30 seconds, 100 µl of the vortexed salivary sample was pipetted out using a standard 100 µl pipette and serial dilutions (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5}) were prepared with sterile 0.85% sterile saline. Using a sterile spreader 100 µl volume from each of the dilutions was pipetted and spread onto the surface of Trypticase yeast cystine agar supplemented with 20% sucrose, Bacitracin 0.2 units /ml, Colistin 10 micrograms /ml and Nystatin 30 micrograms /ml in duplicates and incubated at 37^0c for 48 hours in a 5% CO_2 enriched atmosphere. Colonies morphologically resembling *Streptococcus mutans* were identified by grams reaction, biochemical reactions and biofilm formation. Colonies conforming to *Streptococcus mutans* were counted and total count per ml of saliva was derived by multiplying the number of colonies with dilution factor. Counts are expressed as Log10 transformed colony forming units /ml. Representative colonies of *Streptococcus mutans* were maintained in Brain Heart Infusion medium with 3% glycerol at -20^0C for further studies.

CELL ADHERENCE TO GLASS SURFACE ASSAY

Cell adherence to glass surface assay was performed by the method of (Hamada and Torii 1978) with slight modifications. The bacteria were cultured in 5 ml BHI overnight at 37^0C from stocks kept frozen

to produce log-phase cells. 0.5 ml of the suspensions adjusted to 0.5 on McFarland scale was transferred to a glass tube with 10 ml of BHI with 5% sucrose. The bacteria were grown for 24 h at 37^0 C at an angle of 30 degree. After incubation, planktonic cells were decanted, and the adherent cells were gently washed three times with 2.0 ml of saline. The adherent cells were removed from the glass surfaces by the addition of 0.5 N NaOH (3.0 ml) followed by rapid agitation. Adherence was quantified by reading at 540 nm and % of adherence was calculated for the representative isolates of the participants. Standard strain *Streptococcus mutans* serotype c MTCC (497) obtained from Depository at Chandigarh in India as a lyophilized culture was used as control and simultaneously the absorbances were corrected for non-sucrose-dependent cell adherence. Adherence is the proportion of spectrophotometric measurement (at 540nm) of cells adhering to glass as a percentage of total cell density. Experiments were put up in duplicates.

Preparation of cell-associated glucosyltransferase (Horikoshi *et al*., 1995)

The *Streptococcus mutans* strains were cultivated onto 150 ml of Brain Heart Infusion broth with 1% sucrose and it was incubated for 18 hours at 37^0C and the cultured cells thus obtained were then centrifuged at 10000 rpm for 15 minutes and the pellet was washed twice with saline. Then, the washed cells were suspended in 4 ml of 8M urea solution and the cell suspension was stirred at 25.degree.C for 1 hour. The cell suspension was then centrifuged to remove the cells and the resultant supernatant was dialyzed against10 mM phosphate buffer (pH 6.0). After the dialysis, the precipitate produced in the supernatant was removed by centrifugation and the resultant supernatant was precipitated with ammonium sulfate at 60% saturation. The obtained precipitate was recovered by centrifugation. The precipitate was then dissolved in 10 mM phosphate buffer (pH 6.0) and the resultant solution was dialyzed against the same buffer. Further, the precipitate appeared in the solution was removed by centrifugation to obtain a supernatant. The supernatant was used as a crude antigen. Protein content was determined by Lowry method using bovine serum albumin (BSA) as the standard and characterization of CA-GTF antigen was done by SDS-PAGE (Laemmli, 1970).

GTFs activity (Nezar *et al*., 2005)

About 3ml of sodium acetate buffer (100mM; pH 6.2), 0.5 ml of potassium phosphate buffer and 5% sucrose were added to1.5ml of crude bacterial enzyme. The crude mixture was incubated at 37 degree C for 2 hours. The water insoluble glucan was separated by centrifugation at 10,000g for 5 minutes and it was quantified by phenol sulphuric acid method (Dubois *et al*. 1956). To 50 µL of WIS glucan suspension, 50 µL of deionized distilled water was added and this was followed by the addition of 50 µL of phenol (80% w/v). The mixture was vortexed and subsequently 2 ml of concentrated sulfuric acid was added to the mixture. This mixture was then allowed to stand for 10 minutes at room temperature before its optical density at 490 nm was measured. For the standards, a range of volumes (0-100 µl) of the stock glucose was prepared to give a range of final concentration from 0 to 1 mg /ml. For the blank control, 100 µl of deionized distilled water was used instead. The determinations were carried out in triplicates. The glucose content determined corresponds to the amount of extracellular polysaccharide formed by the partially purifed GTF within the specifc time which was then used in the calculation of the GTF activity. The enzyme activity is expressed as µmol glucose in the extracellular polysaccharide produced per min (µmole glucose min-1) (Rahim and Khan, 2006).

STATISTICALANALYSIS

Obtained data from the study were presented as means and standard values. Significant level was set at 1% for all analyses. Independent variables were WIG synthesis, salivary *Streptococcus mutans* count and sucrose dependent adherence to glass surface and dependant variables were DMFT and DMFS. Statistical analysis was done using student t test for comparing the means of the results of the two groups. Before applying t test the variances of both the groups was tested using Levene's test. Dental caries experience was correlated with sucrose dependent adherence, insoluble glucan production and salivary *Streptococcus mutans* count using Pearson's correlation coefficient. Multiple regression was applied to study the effect of salivary mutans count .sucrose dependent adherence to glass and WIG production on the probability of occurrence of caries activity. The data were statistically analyzed using ANOVA test. The recorded data were transferred to an MS-excel sheet and statistical analysis was carried out using Statistical Package for Social Sciences (SPSS application version 16.)

RESULTS

Streptococcus mutans was isolated from all the samples. The mean value of DMFT of group A having range of 3 to 8 was 5.58 and DMFS having a range of 13 to 24 was 17.3 and group B (Control group) had 0 DMFT and 0 DMFS. The mean value of *Streptococcus mutans* count of group A was 8.61x105 and for the control group was 6.57x104. Water insoluble glucan synthesis mean values were 6.7 and 0.43 for group A and group B respectively. Adherence to glass surface mean value of group A was 29.1 and group B was 3.5 The above results indicate that there is a significant difference in the mean values of group A and group B with relevance to *Streptococcus mutans* count, adherence to glass surface and water insoluble glucan synthesis. For comparing the means of the 2 groups, student t test was applied. Before applying t-test, the variances of both the groups was tested using Levens test. With reference to *Streptococcus mutans* count F value was found to be 16.511 which is significant at 1% level and t statistics value was found to be 14.759 which is also significant at 1% level. With respect to adherence to glass surface F value was found to be 19.491 which is significant at 1% level and t statistics value was 24.461 and is also significant at 1% level. For water insoluble glucan synthesis F value was 15.712 which is significant at 1% level and t statistics value was 24.192 and is also significant at 1% level. The above results shows that there is a significant difference between the caries group and caries free group with respect to *Streptococcus mutans* count, adherence to glass surface and water insoluble glucan synthesis indicating a positive association between caries experience and *Streptococcus mutans* count, WIG synthesis and sucrose dependent adherence to glass.

Multiple regressions were done to find out the effects of predictable factors on DMFT and DMFS. Multiple regression equation was fitted by taking the variable DMFT as a dependent variation and *Streptococcus mutans* count, adherence to glass surface and water insoluble glucan synthesis as independent variables. The variables adherence to glass surface and water insoluble glucan synthesis were found to be significant at 1% level and the *Streptococcus mutans* count was not found to be significant. For a unit increase in adherence to glass surface the DMFT value will be increased by 0.108 units when other variables are kept constant. Similarly for a unit increase in water insoluble glucan synthesis DMFT value is increased by 0.273 when the other variables are kept constant. All the three variables together contribute 91.3% to DMFT. R square value when tested for its significance by applying ANOVAs technique F statistics value was 196.41 and found to be highly significant. Hence it can be concluded that R square is significant and the regression model is as adequate one. Out of the two significant variables adherence, and water insoluble glucan synthesis the variable adherence to glass surface was found to be more important than water insoluble glucan synthesis based on Beta value which is 0.579 for adherence to glass surface and 0.367.for water insoluble glucan synthesis.

Another multiple regression equation was fitted by DMFS as a dependent variable and adherence to glass surface and water insoluble glucan synthesis as independent variables. The variables *Streptococcus mutans* count and water insoluble glucan synthesis was found to be significant at 1% level and adherence to glass surface was found to be not significant. For a unit increase in *Streptococcus mutans* count DMFS value will be increased by 2.1451X10-6 units and for a unit increase in water insoluble glucan synthesis DMFS value will be increased by 1.309 units when other variables are kept constant. R square value was fond to be 78.6% which is the contribution of all the three independent variables to the dependent variable DMFS. F statistics value was 68.777 and was found to be significant at 1% level. Hence the contribution 78.6% is significant and regression model is adequate one. Beta value (0.837) shows that water insoluble glucan synthesis is the most important variable in determining DMFS followed by *Streptococcus mutans* count which has the Beta value of 0.285.

Correlation of Dental caries experience with *S. mutans* levels ,WIG synthesis and Adherence to glass surface

	Group A	Group B
DMFT	5.58	0
DMFS	17.3	0
S.mutans count x 10^5	8.6	0.65
Adherence to glass in %	29	3.5
WIG synthesis **µmol/minx10^2**	6.71	0.43

DISCUSSION

Parampreet Pannu *et al*., 2013 in their study have shown a positive association between caries experience and salivary *S. mutans* scores. Dental caries has been strongly associated with mutans streptococci, particularly *Streptococcus mutans*. Many studies have shown a correlation between the occurrences of high salivary *Streptococcus mutans* count and the carious process and counts of *Streptococcus mutans* have been used to monitor caries risk (Kneist et al., 1999). Hence, new caries lesions will develop if high bacterial counts have been recorded (Kristofferson et al., 1985). Therefore, the evaluation of caries risk enables a person to improve hygiene, diet, and an early control of the bacterial counts which may contribute to a decrease in caries development in the long run (Axelsson 1994). Strains from individuals with caries showed high level of sucrose dependent adherence than from no caries individuals with relevance to DMFT. There is a significant correlation between the DMFT index and the number of *S. mutans* cfu, between DMFT and adherence to glass surface, with a stronger correlation between water insoluble glucan synthesis ability and caries activity. In the present study all had *S. mutans* in their saliva including caries negative individuals. Caries individuals with mean DMFT of 5.58 and DMFS 17.3 had mean *S.mutans* count of $8.61x10^5$ but in Caries negative population with 0 DMFT and DMFS had mean *S.mutans* count of $6.57x10^4$ thus increasing the possibility of true correlation between mutans count and caries status . The present study result with relevance to *S.mutans* count is contradictory to some other study reports, in which levels of *Streptococcus mutans* were not associated with high caries experience (Van Palenstein Helderman et al., 1996; Giacaman *et al*., 2010). The results of the present study is in accordance to a certain extent with some previous studies whohave reported a positive correlation between the concentration of mutans streptococci in saliva and dental caries The intensities of WIG bands were positively correlated with caries incidence ($p < 0.05$) and with the ability of to adhere to glass surfaces ($p < .05$). (Lenander-Lumikari *et al*., 2000; Salonen *et al*., 1990). The ability to synthesize WIG is an important virulence factor in initial caries development by increasing the adherence and accumulation of *Streptococcus mutans* in the plaque of young children (Mattos-Graner, 2000). In the sucrose-containing circumstance, the sucrose dependent cell adhesive ability of the Streptococcus mutans isolated from the caries population was significantly higher than that from the caries-free subjects. This indicated that the adhesive ability is related to the caries-causing tendency. In regression analysis *Streptocous mutans* count was not much significant in relation to DMFT but was significant in relation to DMFS. DMFS refers to decayed missing, and filled surfaces whereas DMFT refers to decayed ,missing and filled teeth and DMFS is more reliable specific dependable variable than DMFT as it shows severity of caries and therefore water insoluble glucan synthesis by

Group statistics

	Group	N	Mean	Std. Deviation	Std. Error Mean
Age	A	60	22.95	0.476	0.061
	B	10	22.82	0.333	0.105
Sex	A	60	1.62	0.490	0.063
	B	10	1.70	0.483	0.153
DMFT	A	60	5.58	1.476	0.191
	B	10	.00	.000	0.000
DMFS	A	60	17.30	3.099	0.400
	B	10	.00	.000	.000
Stretococcus mutans count	A	60	8.616667E5	4.1210566E5	5.3202612E4
	B	10	6.570000E4	2.7948564E4	8.8381119E3
Adherence to glass	A	60	29.0933	7.94240	1.02536
	B	10	3.5000	.65828	.20817
WI-Glucansynthesis	A	60	6.7183	1.98170	.25584
	B	10	.4320	.14382	.04548

Streptococcus mutans is the best indicator in preference to *Streptococcus mutans* count for determining future caries progression and for prevention action in young adults.

CONCLUSION

There is a strong correlation between CA-GTF activity and caries experience with the positive significant correlation between DMFS and *Streptococcus mutans* count. Sucrose dependent adhesive ability to the glass surface of the *Streptococcus mutans* isolated from the caries adults was also significantly higher than that from the caries-free adults. CA-GTF activity is the best reliable predictor for presuming further progression of caries rather than *Streptococus mutans* count.

Table 2: Correlation of Dental caries experience with *S. mutans* levels ,WIG synthesis and Adherence to glass surface using Levene's and t Test

	Levene's test		t-test for Equality of Means						
								95% Confidence Interval of the Difference	
	F	Sig.	T	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Age	1.551	0.217	0.82	68	.411	.130	.157	-.183	.443
			1.06	15.90	.302	.130	.122	-.128	.388
Sex	1.465	0.230	-.49	68	.620	-.083	.167	-.417	.250
			-.50	12.30	.623	-.083	.165	-.443	.276
DMFT	36.982	0	11.87	68	0	5.583	.470	4.646	6.521
			29.26	59.00		5.583	.191	5.202	5.965
DMFS	25.382	0	17.54	68	0	17.3	.986	15.333	19.267
			43.22	59.00		17.3	.400	16.499	18.101
Streptococcus mutans count	16.511	0	6.06	68	0	7.95	1.31	5.34	1.05
			14.75	61.92		7.95	5.39	6.88	9.03
Adherence to glass	19.491	0	10.13	68	0	25.59	2.52	20.54	30.63
			24.46	63.25		25.59	1.04	23.50	27.68
WIG synthesis	15.712	0	9.96	68	0	6.28	.63075	5.02769	7.54498
			24.19	62.38		6.28633	.25985	5.76697	6.80570

Coefficientsa

Model		Unstandardized Coefficients		Standardized Coefficients	T	Sig.
		B	Std. Error	Beta		
1	(Constant)	.515	.229		2.247	.029
	Smcount	1.172E-7	.000	.033	.532	.597
	Adherence	.108	.021	.579	5.184	.000
	WinsGlucansyn	.273	.082	.367	3.352	.001

a. Dependent Variable: DMFT

Coefficientsa

Model		Unstandardized Coefficients		Standardized Coefficients	T	Sig.
		B	Std. Error	Beta		
1	(Constant)	8.889	.755		11.775	.000
	Smcount	2.145E-6	.000	.285	2.955	.005
	Adherence	-.077	.068	-.197	-1.122	.267
	WinsGlucansyn	1.309	.268	.837	4.878	.000

a. Dependent Variable: DMFS

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