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Journal of the Dental Association of South Africa, **47,** 333-336.

Plaque quantitation through protein measurement

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Keywords: dental plaque; standardisation; protein content.

SUMMARY

This study was undertaken to establish whether the quantitation of dental plaque protein by a dye-binding method (Coomassie G-250) may be used as an index of the amount of dental plaque sampled. Ten sites were sampled in 34 children on 5 occasions at 4 month intervals. The mean protein concentration in 1391 plaque samples was 6,9 ± 4,1 micrograms (µg) (mean ± standard deviation). A three-way analysis of variance showed that the plaque protein concentration was similar at the different sampling sites in the same child (p=0,14), but statistically significant differences were observed with respect to time of sampling (F=36,24; p=0,0001) and individual sampled (F=5,69; p=0,0001). These observations indicate that plaque bacterial counts may be expressed as units of protein concentration and this method may be useful to relate the number of viable bacteria to an estimate of the amount of plaque collected. This ratio allows standardisation for any variation in the amount of plaque collected.

OPSOMMING

Die doel van hierdie studie is om vas te stel of die proteïeninhoud van tandheelkundige plaakmonsters 'n aanduiding van monstergroote lewer, indien 'n kleurstofbindingsmetode (Coomassie G-250)) toegepas word. Monsters is van 10 tandoppervlakke in 34 kinders tydens vyf geleenthede op 'n viermaandelikse grondslag verkry. Die proteïenkonsentrasie in 1391 plaakmonsters was 6,9 + 4,1 mikrogram (µg) (gemiddelde + standaardafwyking).'n Drieringting analise van variansie het getoon dat daar geen statisties betekenisvolle verskille tussen die oppervlakke in 'n gegewe mond is nie (p=0,14). Betekenisvolle verskille is waargeneem ten opsigte van ondersoektydperk (F=36,24; p=0,0001) en indivïdue (F=5,69; p=0,0001). Hierdie resultate het getoon dat die verhouding van die aantal lewensvatbare bakterie tot die proteïeninhoud dit moontlik maak om te standardiseer ten opsigte van enige variasie in die grootte van die monster.

INTRODUCTION

The microbiology of dental plaque has been extensively studied in an attempt to understand dental caries (Bush, Challancombe and Newman, 1990; Mundorf *et al.,* 1990) and periodontal disease (Newman, 1990). In these investigations results are reported as number of viable bacterial counts or the colony forming units (CFU). Meaningful interpretation of the findings is only possible when the counts are related to the amount of plaque obtained, since the plaque mass has some influence on the number of CFU (Gilmour, Zahn and Turner, 1978). Unfortunately, there is a poor correlation between the number of viable *Streptococcus mutans* organisms and the wet weight of plaque due to the extremely variable structure of plaque (Distler, Petschelt and Kröhnke, 1987). Studies (Mandel, 1974; Gilmour *et al.,* 1978) have been undertaken to address the problem of quantifying plaque in some way and relating it to viable bacterial numbers. Unfortunately, most of the procedures followed are time-consuming and expensive. Moreover, they are only useful in investigations of (pooled) plaque samples with a relatively high mass, and are not suitable for studies of individual plaque samples (Mandel, 1974). In caries investigations it is more appropriate to analyse individual than pooled plaque samples, since caries is a local process (Bowen, 1987).

Another approach is to relate the number of viable bacteria in plaque to its protein concentration in micrograms (µg) in a similar manner to the expression of the specific activity of enzymes per milligram of protein (Metzler, 1977). Jones *et al.,* (1988) followed this approach by using a simple dye-binding method, developed by Bradford (1976) and modified by Brogdon and Dickinson (1983) in a dentifrice field study in which plaque growth was measured twice: at baseline and after 2 years. They reported their results as the total viable bacterial count per microgram of protein concentration but did not state actual values for the protein concentration of the individual plaque samples.

The present study was undertaken to evaluate a modified Coomassie G-250 dye-binding method (Bradford, 1976) for protein estimation of dental plaque and to relate this to the total number of viable bacteria.

MATERIALS AND METHODS

Prior to undertaking the study the research protocol was approved by the Committee for Research on Human Subjects of the University of the Witwatersrand, Johannesburg.

Protein Assay

The protein concentration was measured by the Coomassie G-250 dye-binding method described by Bradford (1976). This assay is based on the colour change of a dye in response to concentrations of protein. The assay was modified by changing the sample : reagent ratio from 1 : 10 to 1 : 1.

The spectrophotometer (Pye-Unicam, Cambridge, England) was zeroed against a reagent blank, containing reduced transport fluid (RTF) (Syed and Loesche, 1972) and the Coomassie reagent. Bovine serum albumin, dissolved in RTF, was used as a protein containing solution for the

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Article received: 28:6:91
approved for publication: 23:2:92

standardisation of the dental plaque protein concentration. A standard curve was constructed by plotting the spectrophotometric absorbance against the albumin protein concentration over the range 0 - 10 μg in 2,5 μg increments.

Assay reproducibility was evaluated by determining the intra-assay coefficient of variation on 10 identical samples, while the inter-assay coefficient of variation was determined on 5 occasions on the same pooled sample as recommended by Lloyd (1978).

Dental Plaque Samples

Dental plaque samples from 10 different sites were obtained from 34 nursery school children on 5 occasions at approximately 4 month intervals. A total of 1391 samples were obtained out of a possible 1700, since some children were not present at all 5 visits or some of the teeth were missing. Samples were obtained by scraping teeth with a sterile toothpick after which the tips of the tooth pick were cut off and dropped into bottles containing 2 ml reduced transport fluid (RTF) (Syed and Loesche, 1972). Four samples were taken from anterior teeth: the buccal and distal surfaces of incisors (teeth 61 and 62) and 6 from posterior teeth: occlusal and mesial surfaces of molars (teeth 64, 65, 74 and 75). Six surfaces were smooth while the remaining 4 surfaces were occlusal. An aliquot, 0,2 ml, of the plaque sample was removed from the inoculated RTF for microbiological analysis, while the remainder was stored at -20° C until the protein content was determined.

Total Viable Bacterial Counts

The total viable anaerobic bacterial count for each plaque sample was recorded by counting the colony forming units (CFU) of a ten-fold dilution (in peptone water) on blood agar plates.

Protein Estimation in Plaque Samples

Plaque samples from the same individual for a particular visit (sampling time) were analysed in the same run to eliminate the effect of inter-assay variation.

Bacterial Load on Teeth

The number of colony forming units per microgram of protein (CFU/μg protein) was calculated to assess the bacterial load on teeth (Jones *et al.,* 1988)

Statistical Analyses

SAS (1986) on an IBM 3083 J24 mainframe computer was used to evaluate results. The means for the protein concentration, CFU and CFU/μg were calculated. The Pearson correlation coefficient for the protein concentration and number of colony forming units was determined. A 3-way analysis of variance (ANOVA) was performed with the protein concentration, the number of CFU or the CFU/μg protein (the dependent variable) and individual child, sampling time and sampling site (the independent variables). The critical level of statistical significance chosen was p<0,05.

RESULTS

Protein Assay

The volume modifications had no effect on the background absorbance as it was equal to 0,5 - 0,6 absorbance units in both our modification and in Bradford's (1976) original micromethod. However, the modification of the sample : reagent ratio lowered the limit of detection from 10 to 2,5 μg. A linear standard curve, fitting the equation, concentration = 23,2 ∞ absorbance + 0,029 was obtained for the 1 : 1 ratio, compared to a horizontal line equal to approximately 0,135 absorbance units over the concentration range 2,5 - 10 μg, when the original 1 : 10 sample to reagent ratio was used.

Precipitation was noted in the reagent blank, standards and samples after approximately 45 minutes, resulting in a decrease in absorbance by approximately 25 per cent. This problem was overcome by limiting the number of samples to be analysed to a number which could be analysed in less than this precipitation period.

The intra-assay coefficient of variation was 17 per cent [3,5 ± 0,6 microgram protein (mean ± standard deviation)] for 10 identical samples, while the inter-assay variation in 5 consecutive runs was 20 per cent (2,9 ± 0,6 μg protein).

The Protein Concentration of Dental Plaque Samples

The mean protein concentration in 1391 plaque samples was 6,9 ± 4,1 microgram The observed minimum was less than the limit of detection (<2,5 μg), while the maximum was 45 micrograms. Mean values for the sampling sites and sampling times are summarised in tables I and II respectively. There was no statistically difference between the sites sampled in the same subject (ANOVA F=1,51; p=0,14). Statistically significant differences were, however, observed between measurements with respect to the time of sampling (ANOVA F=36,24; p=0,0001) and the individual sampled (ANOVA F=5,69; p=0,0001).

Table I: *The number of samples (n), mean, standard deviation (sd), minimum and maximum for the protein concentration in micrograms in plaque samples collected from various teeth and surfaces (sites).*
B: buccal, D: distal O: occlusal, M: mesial.

Site	n	Mean	sd	Minimum	Maximum
61B	137	6,8	4,6	<2,5	43
61D	139	6,5	4,3	<2,5	29
62B	140	7,0	4,3	<2,5	19
62D	139	6,3	3,2	<2,5	16
64O	141	6,5	4,4	<2,5	32
65O	139	7,3	4,0	<2,5	26
74M	139	6,9	3,6	<2,5	17
74O	139	7,3	4,0	<2,5	26
75M	139	7,5	5,3	<2,5	45
75O	226	6,8	3,6	<2,5	19

Table II: *The number of samples (n), mean, standard deviation (sd), minimum and maximum for the protein concentration (μg) in plaque samples obtained on various occasions (visits).*

Visit	n	Mean	sd	Minimum	Maximum
1	283	8,3	3,0	<2,5	45
2	325	6,5	4,5	<2,5	43
3	325	7,8	4,6	<2,5	29
4	258	6,5	3,6	<2,5	32
5	200	4,5	2,2	<2,5	20

The Number of Colony Forming Units in Dental Plaque Samples

The mean number of CFU in 1514 plaque samples was $4,7 \infty 10^6 \pm 1,3 \infty 10^6$ colony forming units (CFU). The minimum was $1,2 \infty 10^3$ CFU, while the maximum was $2,2 \infty 10^8$ CFU. The limit of detection is $1 \infty 10^2$ CFU. Mean values for the sampling sites and sampling times are summarised in tables III and IV

respectively. Statistically significant differences were observed with respect to the sampling site (ANOVA F=11,73; p=0,0001), sampling time (ANOVA F=8,45; p=0,0001) and individual child sampled (ANOVA F=2,82; p=0,0001).

Table III: *The number of samples (n), mean, standard deviation (sd), minimum and maximum for the number of viable bacteria (CFU) in plaque samples collected from various teeth and surfaces (sites). B: buccal, D: distal O: occlusal, M: mesial.*

Site	n	Mean	sd	Minimum	Maximum
61B	149	$7,0\infty10^{6}$	$1,8\infty10^{7}$	$1,4\infty10^{3}$	$2,0\infty10^{8}$
61D	149	$3,9\infty10^{6}$	$7,0\infty10^{6}$	$1,4\infty10^{3}$	$5,6\infty10^{7}$
62B	150	$1,3\infty10^{7}$	$2,7\infty10^{7}$	$1,2\infty10^{3}$	$2,2\infty10^{8}$
62D	153	$7,7\infty10^{6}$	$1,7\infty10^{7}$	$1,2\infty10^{3}$	$1,2\infty10^{8}$
64O	152	$1,4\infty10^{6}$	$3,3\infty10^{6}$	$1,9\infty10^{3}$	$2,9\infty10^{8}$
65O	153	$2,3\infty10^{6}$	$6,0\infty10^{6}$	$1,4\infty10^{3}$	$4,2\infty10^{7}$
74M	150	$4,0\infty10^{6}$	$9,6\infty10^{6}$	$2,0\infty10^{3}$	$7,5\infty10^{7}$
74O	152	$2,6\infty10^{6}$	$4,1\infty10^{6}$	$1,9\infty10^{3}$	$3,6\infty10^{7}$
75M	153	$4,2\infty10^{6}$	$9,3\infty10^{6}$	$2,0\infty10^{3}$	$5,6\infty10^{7}$
75O	153	$2,1\infty10^{6}$	$4,6\infty10^{6}$	$4,0\infty10^{3}$	$2,8\infty10^{7}$

Table IV: *The number of samples (n), mean, standard deviation (sd), minimum and maximum for the number of viable bacteria (CFU) in plaque samples obtained on five occasions.*

Visit	n	Mean	sd	Minimum	Maximum
1	324	$8,0\infty10^{6}$	$2,3\infty10^{7}$	$1,9\infty10^{3}$	$2,2\infty10^{8}$
2	329	$3,5\infty10^{6}$	$7,3\infty10^{6}$	$1,9\infty10^{3}$	$7,2\infty10^{7}$
3	327	$3,1\infty10^{6}$	$6,6\infty10^{6}$	$1,9\infty10^{3}$	$5,6\infty10^{7}$
4	255	$3,5\infty10^{6}$	$8,5\infty10^{6}$	$1,2\infty10^{3}$	$6,4\infty10^{7}$
5	199	$5,8\infty10^{6}$	$1,3\infty10^{7}$	$2,0\infty10^{6}$	$1,4\infty10^{8}$

The Correlation of the Protein Concentration with the number of CFU

The correlation of the protein concentration with the total number of viable bacteria was low, only r=0,10. This was statistically significant (p=0,0004) but is due to the large sample size and should be disregarded.

The Bacterial Load on Teeth

The mean total viable bacterial counts expressed in terms of the protein concentration (CFU/µg protein) in 1377 plaque samples was $1,1\infty10^{6}\pm6,9\infty10^{6}$ CFU/µg protein. The minimum was 118, while the maximum was $2,3\infty10^{8}$ CFU/µg protein. Mean values for the sampling sites and sampling times are summarised in tables V and VI respectively. The bacterial load on teeth did not vary between teeth (ANOVA F=0,86; p=0,4889) and individual child, (ANOVA F=1,24; p=0,1678) but differences were observed with regard to sampling site (ANOVA F=4,13; p=0,001).

Table V: *The number of samples (n), mean, standard deviation (sd), minimum and maximum for the number of viable bacteria per microgram of protein (CFU/µg) in plaque samples collected from various teeth and surfaces (sites). B: buccal, D: distal O: occlusal, M: mesial.*

Site	n	Mean	sd	Minimum	Maximum
61B	134	$1,7\infty10^{6}$	$6,0\infty10^{6}$	204	$6,6\infty10^{7}$
61D	137	$1,0\infty10^{6}$	$2,0\infty10^{6}$	141	$1,4\infty10^{7}$
62B	138	$4,2\infty10^{6}$	$2,0\infty10^{7}$	137	$2,3\infty10^{8}$
62D	136	$1,4\infty10^{6}$	$2,8\infty10^{6}$	212	$2,1\infty10^{7}$
64O	140	$2,9\infty10^{5}$	$5,7\infty10^{5}$	222	$3,0\infty10^{6}$
65O	138	$5,1\infty10^{5}$	$1,6\infty10^{6}$	191	$1,5\infty10^{8}$
74M	136	$5,4\infty10^{5}$	$1,4\infty10^{6}$	127	$8,0\infty10^{6}$
74O	140	$3,4\infty10^{5}$	$8,6\infty10^{5}$	197	$7,2\infty10^{6}$
75M	139	$7,0\infty10^{5}$	$1,4\infty10^{6}$	118	$9,6\infty10^{6}$
75O	139	$6,7\infty10^{5}$	$2,7\infty10^{6}$	449	$2,8\infty10^{7}$

Table VI: *The number of samples (n), mean, standard deviation (sd), minimum and maximum for the number of viable bacteria per micrograms of protein (CFU/µg) in plaque samples obtained on various occasions (visits).*

Visit	n	Mean	sd	Minimum	Maximum
1	281	$6,8\infty10^{5}$	$1,6\infty10^{5}$	197	$1,9\infty10^{7}$
2	325	$1,5\infty10^{6}$	$1,3\infty10^{7}$	333	$2,3\infty10^{8}$
3	322	$1,0\infty10^{6}$	$4,3\infty10^{6}$	118	$6,6\infty10^{7}$
4	251	$9,2\infty10^{5}$	$2,9\infty10^{6}$	137	$2,8\infty10^{7}$
5	198	$1,4\infty10^{6}$	$2,7\infty10^{6}$	588	$2,4\infty10^{7}$

DISCUSSION

The Coomassie dye-binding method is convenient when samples are to be analysed in large batches as it involves a single dilution step and is suitable for the detection of protein at the low concentrations encountered in individual dental plaque samples.

The coefficients of variation obtained in the present study appeared to be high (17 - 20 per cent) but it should be remembered that a small variation in a low concentration may appear large compared to the same variation in a higher concentration. It is, therefore, tenable in situations like the present, where protein is present in a low concentration, to find that variations are relatively large.

The Lowrey *et al.,* (1951) protein assay which is regarded as the gold standard for the micro-determination of protein have been unsuitable for the present study because the reduced transport fluid would have interfered with the assay by producing a high background photospectrometric absorbance. It was thus impossible to compare this method to the one we used. It is also not possible to compare results obtained by dye-binding in the present study to those obtained by Distler *et al.,* (1987) using gas chromatography since different calibration standards were used.

The analysis of variance (ANOVA) showed that the plaque protein content is similar at different sites in a given mouth at a particular moment in time, but significantly different between individual children at different intervals.

The lack of correlation of the number of CFU with the protein concentration was not surprising, since the protein in dental plaque is not only derived from bacteria, but also from the diet and biological fluids (Laird and Grant, 1983).

The analyses of variance (ANOVA) showed that the number of colony forming units (CFU) varied from site to site, visit to visit and individual to individual, whereas the bacterial load (CFU/µg protein) varied only from site to site. It may be deduced from these results that the bacterial load, or the number of bacteria per microgram of plaque protein, may appear similar between individuals and constant between sampling intervals. However, within the same mouth, the same amount of plaque may give rise to variable numbers of viable bacteria at different sites. These observations suggest that the number of CFU within dental plaque vary relative to the plaque protein content. If the protein content is used to standardise for the different sampling sites in a mouth, then it will be possible to see what variations there are in CFU from site to site.

Although the Coomassie dye-binding method has some deficiencies, it appears at present the most practical solution to the problem of standardising bacteriological counts in terms of the amount of plaque obtained from a particular sampling site.

ACKNOWLEDGEMENTS

We are grateful to Dr Carol Jones, Unilever Research, UK for help with this study and Prof LP Fatti, Department of Statistics and Applied Mathematics, University of the Witwatersrand, Johannesburg for advice on the statistical analyses.

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