Oral Microbiology Immunology 2007: 22: 61–66
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ORAL MICROBIOLOGY
AND IMMUNOLOGY

The relationship between dental caries status and dental plaque urease activity

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Shu M, Morou-Bermudez E, Suárez-Pérez E, Rivera-Miranda C, Browngardt CM, Chen Y-YM, Magnusson I, Burne RA. The relationship between dental caries status and dental plaque urease activity.
Oral Microbiol Imunol 2007: 22: 61–66. © 2007 The Authors. Journal compilation © 2007 Blackwell Munksgaard.

Introduction: Ammonia production from the metabolism of urea by urease enzymes of oral bacteria moderates plaque acidification and may inhibit dental caries, as suggested by *in vitro* studies and indirect clinical observations. The objective of this study was to examine the relationship of urease activity with dental caries at the clinical level.
Methods: Urease activity was measured in dental plaque and saliva samples from 25 caries-free subjects (CF) and in eight subjects with six or more open caries lesions (CA). Plaque and saliva collection was repeated for each subject 1 week later using identical procedures.
Results: Urease-specific activity in the dental plaque of CF subjects was significantly higher compared to that in the subjects with caries. The association of low plaque urease levels with increased caries was further supported by odds ratio analysis using different plaque urease cut-off points. Using a receiver operating characteristic curve it was estimated that there was an approximately 85% probability of correctly classifying the subjects as CA or CF based on the relative ordering of their plaque urease activity levels. No statistically significant differences were observed in salivary urease activity.
Conclusion: This study suggests that loss of alkali-generating potential of tooth biofilms via the urease pathway has a positive relationship to dental caries.

Key words: alkali; ammonia; biofilm; dental caries; ecology; urea

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Accepted for publication June 26, 2006

The hydrolysis of urea by bacterial urease enzymes generates ammonia and CO_2 and it is considered a major pathway for alkali production in the oral cavity (3, 27). Urea enters the oral cavity in all salivary secretions and in the gingival crevicular fluid at concentrations ranging between 1 and 10 mM in healthy individuals (1, 14, 18). Numerous studies have shown that urea at concentrations comparable to those normally found in saliva can significantly increase the baseline pH of dental plaque, and that it can effectively counteract the effects of glycolytic acidification on the plaque pH (10, 11, 16, 17, 26, 29). It has, therefore, been hypothesized that the production of ammonia via ureolysis in the oral cavity may be an important factor inhibiting the emergence of a cariogenic flora and the development of caries. Indeed, a link between markedly elevated urea levels in the saliva of renal dialysis patients and caries resistance has been noted (1, 12, 23, 25). More recently, the introduction of the urease gene cluster of *Streptococcus salivarius* into the cariogenic organism *Streptococcus mutans* demonstrated that the ability of these recombinant urease-producing *S. mutans* strains to induce caries formation in specific pathogen-free rats was dramatically reduced compared to the wild-type, non-ureolytic strain (9).

Although there is enough indirect evidence to suggest that ammonia production from bacterial ureolytic activity in the oral cavity may inhibit the development of dental caries, clinical studies directly evaluating the association of urease activity with dental caries have been very few and inconclusive. Hine and O'Donnell (15) noticed a possible inverse association between caries activity and salivary urease activity, although other investigators (2, 22) did not confirm this observation. Frostel in 1960 (13) measured urease levels in plaque from caries-active and caries-inactive human volunteers using an antimony electrode and found no statistically significant differences. Studies performed in the 1940s, 1950s and 1960s employed various methodologies for

defining urease activity and caries activity, and the statistical analysis of the data was limited, or, often omitted. Since then, our knowledge of the biochemistry, genetics and regulation of oral ureases has increased significantly and it provides further support to the concept that production of ammonia from ureolysis in the oral cavity may be an important homeostatic mechanism for pH in dental plaque. It appears, therefore, appropriate and necessary to re-evaluate the magnitude and the direction of the association of urease activity with dental caries at a clinical level using modern and thorough biochemical and statistical approaches. For that purpose we compared the levels of urease activity in the dental plaque and in the saliva of two groups of healthy volunteers, namely a caries-active (CA) group and a caries-free (CF) group. The working hypothesis in the study was that an inverse association may exist between urease activity levels in plaque and/or in saliva, and dental caries in humans.

Materials and methods

Study sample

Twenty-five CF subjects (mean age 24.9 years, 56% female, 44% male) and eight CA subjects (mean age 26.6 years, 62.5% female, 37.5% male) were recruited for this study from the pre-doctoral clinic of the University of Florida. CA subjects had at least six cavitated, unrestored carious lesions, whereas CF subjects were individuals who had no clinical evidence of caries experience in their permanent dentition. All of the 33 subjects had good periodontal health, were in good general health, and none of the subjects was taking antibiotics during the study period. Informed consent was obtained under a protocol reviewed and approved by the University of Florida Health Science Center Institutional Review Board (#41-2002).

Clinical procedures

The participants refrained from toothbrushing for approximately 16 h and had fasted overnight before sample collection. Supra-gingival plaque samples were collected using a periodontal curette from all available tooth surfaces, with the exception of the lingual surfaces of the lower anterior teeth, of one-half of an individual mouth, where a half mouth was defined as the upper and lower quadrant of the same side of the mouth (30). These particular dental surfaces were excluded because they are more exposed to salivary urea compared to the other surfaces and because they are frequently covered with calculus, which makes plaque collection more difficult. Plaque from a single individual was pooled in a microcentrifuge tube containing 0.5 ml 10 mM sodium phosphate buffer (pH 8.0). The tube was kept on ice during sample collection. Approximately 2 ml of whole unstimulated saliva was collected from each participant by asking the subjects to expectorate into a chilled sterile plastic tube (Falcon 2070, Becton Dickinson and Company, Franklin Lakes, NJ). Saliva samples were collected before plaque collection. The plaque and saliva samples were transferred to the laboratory for analysis within 15 min. The same procedure was carried out 1 week later for the other half of the mouth.

Urease activity

Urease activity in the plaque and saliva samples was determined immediately following collection by measuring the amount of ammonia released by urea hydrolysis by fresh plaque and saliva samples. Plaque was resuspended by vortexing for 10 s. A 10-µl aliquot of each of the suspended plaque and saliva samples was incubated with 50 mM urea at 37°C for 120 min in 50 mM potassium phosphate buffer, pH 7.0 (final volume of urease assay: 500 µl). Each plaque and saliva sample was assayed in triplicate identical reactions. The amount of ammonia released was measured using the Nessler Reagent (Aldrich Chemical Company, Inc, Milwaukee, WI) as previously described (8). Ammonium sulfate was used to prepare standard curves. A control reaction without urea was performed for each sample to measure background levels of ammonia from non-urease sources. Urease activity was expressed as µmoles urea hydrolysed/min and it was normalized to either mg of total protein or to 10^7 colony-forming units (CFU $\times$ 10^7).

Microbiological procedures

During the incubation period for the urease assays, suspended plaque and saliva samples were vortexed for 30 s. One portion (50 µl) was serially diluted in 0.5% proteose peptone and 0.25% KCl and plated onto Columbia blood agar (CBA; Columbia blood agar base, Difco, Detroit, MI, plus 5% sheep blood). The plates were incubated at 37°C in an anaerobic chamber (Plas Labs, Inc., Lansing, MI) for 7 days before counting colonies. The remainder of the suspended plaque and saliva samples were stored at −20°C.

Protein determination

Total protein in the samples was measured as follows. The plaque samples were thawed on ice, 250 µl of the sample was mixed with 250 µl H_2O and 500 µl glass beads (0.1-mm, BioSpec Products, Inc., Bartlesville, OK) were added. The samples were then homogenized in a Bead Beater for 30 s, twice, with cooling on ice during the interval. For saliva samples, 500 µl saliva was mixed with 500 µl glass beads and homogenized as above. The samples were centrifuged for 5 min at 13,000 ***g*** in a refrigerated microcentrifuge. The protein concentration of the supernatant fluid was determined using the BioRad Protein assay reagent (BioRad Laboratories, Hercules, CA) with bovine serum albumin as the standard.

Data analysis

Descriptive statistics including means, standard deviations, ranges and box-plots were used to evaluate the distribution of urease levels in each group. Means between the two groups were compared using a linear mixed model on the STATA program (StataCorp LP, College Station, TX) with the GLLAMM (Generalized Linear Latent and Mixed Model) command (31) to adjust for the correlation between the first and second measurements within each subject. Analysis was carried-out using log transformation of the data because of the high variability. Odds ratios were used to quantify the association between urease levels and dental caries using different plaque urease cut-off points. Receiver operating characteristic (ROC) curves (24) were used to quantify the diagnostic accuracy of urease activity as a means to determine the caries status of the subjects. All analysis was performed using the STATA version 9.0 program.

Results

The distribution of plaque urease specific activity levels (expressed as µmoles of urea hydrolysed/min/mg protein) in the CA and the CF groups is presented in Fig. 1A,B, respectively, and in Table 1. Saliva urease activity levels were generally lower than plaque urease levels in both groups. The distribution of both the plaque and the saliva urease activity was fairly asymmetric in both groups because the distance of the observations above the median was larger than that below the median (positively skewed).

Urease activity is presented normalized to either mg total protein or CFU $\times$ 10^7 to reflect the amount of activity expressed by

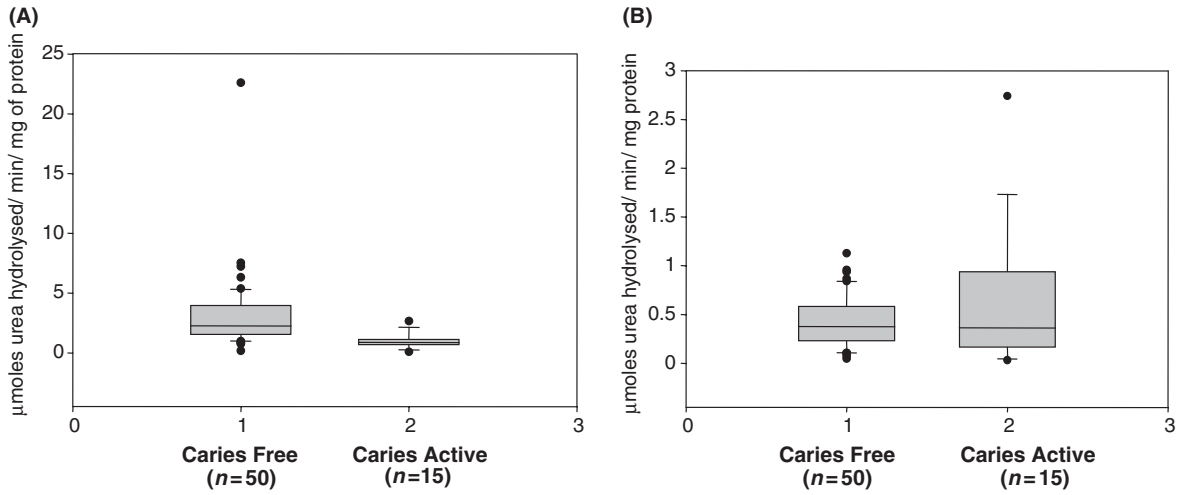

Fig. 1. Distributions of (A) plaque urease activity and (B) saliva urease activity.

Table 1. Comparisons of study variables in the caries-free and caries-active subjects

Variable	Caries-free ($n = 50$) Mean (SE)[1] (min–max)	Caries-active ($n = 15$) Mean (SE)[1] (min–max)	95% CI of the difference[2]	*P*-values[2]
Plaque urease (µmoles/min/mg protein)	3.11 (0.46) (0.19–22.62)	1.0 (0.17) (0.09–2.67)	0.60, 1.55	<0.0001
Plaque urease (µmoles/CFU × 10^7)	3.64 (0.5) (0.43–22.35)	1.72 (0.6) (0.04–7.62)	0.60, 1.80	<0.0001
Saliva urease (µmoles/min/mg protein)	0.41 (0.04) (0.05–1.13)	0.59 (0.18) (0.03–2.74)	−0.69, 0.54	0.8110
Saliva urease (µmoles/CFU × 10^7)	53.2 (5.2) (10.9–150)	85.8 (28.9) (9.9–425)	−0.70, 0.46	0.6950
Plaque protein (µg/half mouth)	188.5 (20.4) (17.8–777.4)	777.6 (115.0) (209.3–1376.4)	−2.06, −0.99	<0.0001
Plaque CFU × 10^7 in half mouth	24.1 (3.2) (1.1–113)	224.2 (96.9) (12–1460)	−2.36, −0.91	<0.0001
Saliva protein (mg/ml)	1.1 (0.05) (0.4–1.9)	1.3 (0.2) (0.6–3.2)	−0.30, 0.21	0.7310
Saliva CFU × 10^7/ml	91.1 (19.6) (3.8–810)	112.4 (31.9) (2–414)	−0.95, 0.71	0.7820

[1]Original values.
[2]Log-transformation is used in the data analysis.

a defined unit of plaque. As presented in Table 1, plaque urease levels differed significantly between the two groups with the urease levels in the CF group being approximately 3-fold higher compared to the CA group when urease activity was normalized to protein ($P < 0.0001$), and 2-fold higher when urease activity was normalized to CFU ($P < 0.0001$). Urease levels in saliva did not differ significantly ($P > 0.05$) between the two groups, regardless of whether they were normalized to protein or to CFU. Background ammonium ion levels in mixed saliva did not differ significantly between the two groups (t-test with equal variance $P = 0.69$). In addition to urease activity, total protein and total cultivable flora were compared in the two groups. CA subjects had significantly more total protein ($P < 0.0001$) and higher CFU numbers ($P < 0.0001$) compared to the CF group. Total protein levels and CFU numbers in saliva did not differ significantly between the two groups.

Plaque urease levels ($n = 65$ observations from a total of 33 subjects) were subsequently divided into quintiles, and the number of observations from CA and CF subjects within each quintile was determined. The sensitivity and specificity of correctly classifying the observations into CA and CF when different plaque urease activity levels were used as cut-off points was estimated (Table 2). As shown in Table 2, when plaque urease levels ≤0.9 units/mg protein (lowest quintile) were used as the cut-off point, 60% of the CA and 92% of the CF observations could be correctly classified. When the cut-off point of plaque urease activity was increased, the probability of identifying the true CA subjects (sensitivity or true positive rate) increased (80% at 1.55-unit cut-off point, 93% at the 2.2-unit cut-off point), while the specificity (true negative rate) decreased. These data were summarized by constructing a ROC curve (Fig. 2). The area under the ROC curve was calculated as 0.8467 (95% CI 0.741, 0.952), which means that there was an 85% probability of correctly distinguishing a CA subject from a CF subject based on the relative ordering of their plaque urease activity levels. Odds ratios were calculated to quantify the association between plaque urease activity and caries status at each level of plaque urease (Table 3). The odds of being CA with plaque urease levels below 0.9 units/mg were about 17 times higher (95% CI: 3.31, 96.67) than the odds of being CA with urease levels above that level. The association between caries status and plaque urease activity was strong at all urease levels, although the 95% confidence intervals were very broad, because of the small number of observations.

Table 2. Sensitivity and specificity of classifying plaque urease observations into caries active and caries free using different plaque urease cut-off points

Plaque urease cut-off point[1] (units/mg protein)	Sensitivity (true positive rate)	Specificity (true negative rate)
≤0.9	60%	92%
≤1.55	80%	72%
≤2.2	93%	50%
≤3.6	100%	26%
>3.6	100%	0

[1]1 unit equals 1 µmol urea hydrolysed/min.

Discussion

Oral bacteria exist as compositionally and structurally complex populations in

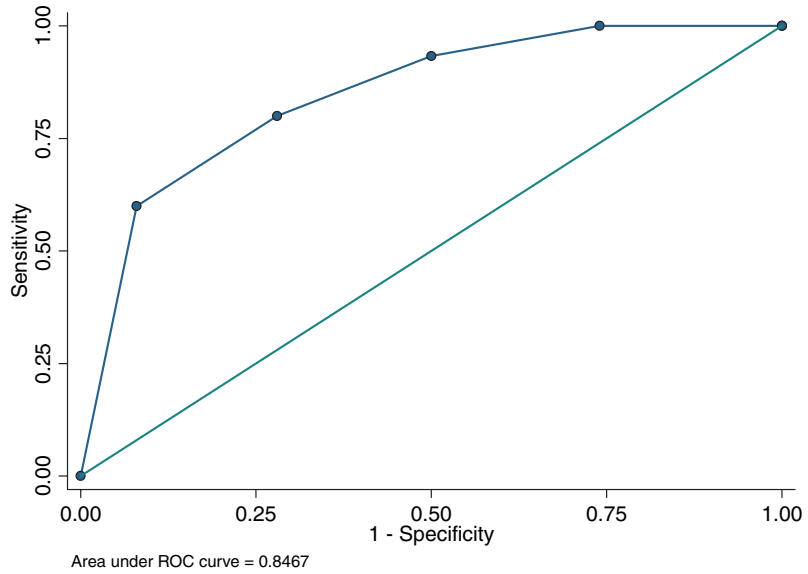

Fig. 2. Receiver operating characteristics (ROC) curve for data on Table 2. Different points on this curve correspond to different plaque urease cut-off points. Number of observations, 65; ROC area, 0.8467; standard error, 0.0539; 95% confidence interval, 0.741, 0.952.

Table 3. Use of different plaque urease cut-off points to estimate the odds ratio of being caries active

Plaque urease cut-off point[1] (units/mg protein)	Odds ratio	95% CI	*P*-value
≤0.9	17.25	3.31, 96.57	<0.0001
≤1.55	10.28	2.2, 62.70	0.0006
≤2.2	14	1.8, 615	0.0025
≤3.6	11.1	1.29, ∞	NC
>3.6	NC	NC	NC

[1]1 unit equals 1 μmol urea hydrolysed/min.

biofilms colonizing the tissues of the mouth. In most cases, oral biofilms are compatible with health. The transition of the oral flora from a commensal to a pathogenic relationship with the host is associated with a change in the metabolism and composition of the flora at specific sites, driven primarily by environmental stresses brought on by changes in diet, salivary flow and other factors (3, 20). The development of dental caries is associated with a significant increase in the proportion of biofilm bacteria that are highly acidogenic and aciduric, such as mutans streptococci, other aciduric streptococci and lactobacilli (4, 5, 32, 33). Consequently, a major focus in caries research has been on identifying and characterizing aciduric bacteria and the molecular mechanisms of acid resistance. One often-overlooked reality is that, concurrent with the increases in the proportions of aciduric organisms in cariogenic plaque, there is a decrease in the proportions of organisms that are generally associated with health. In many cases, the organisms that are lost are those bacteria that are capable of significant alkali production (6).

In the present study, significantly lower levels of urease activity were observed in the dental plaque of CA individuals compared to the CF subjects. It was also determined that, depending on what plaque urease levels were chosen as the cut-off point, the probability of being caries-active was from 10- to 17-times higher for individuals with urease levels below that point, compared to those with urease levels above the cut-off point. Frostel in 1960 (13) also observed higher urease levels in the plaque of caries-free subjects compared to a caries-active group but found this difference to be not statistically significant. The difference between Dr Frostel's results and ours could be the different methodologies employed. Frostel used a titration method for measuring ammonia and normalized the urease activity levels to wet plaque weight. In the present study, a colorimetric assay capable of detecting <10 nmol NH_4 was used and urease activity was normalized either to total plaque protein or to total plaque CFU.

Urease activity in saliva did not appear to differ significantly between CA and CF subjects in the present study. This observation is in accordance with previous reports (2, 13, 22). The fact that salivary urease levels are not different among the caries groups suggests that the relationship between ureolytic activity and caries in the two populations of subjects may not be the result of enhanced competition between organisms in plaque and saliva for the substrate. The fact that a significant association between urease levels and dental caries levels was observed in plaque but not in saliva is not surprising. Given the site-specific nature of caries, one would not necessarily predict that the biochemical activities of salivary populations would necessarily reflect those of plaque. In fact, a recent study by Socransky et al. (19) demonstrated substantial differences in plaque and salivary populations. Collectively then, it does not seem that a rapid diagnostic system that is based on measuring urease or ammonia in saliva would be a reasonable predictor of caries susceptibility or activity, although this is an attractive concept that may warrant more investigation because of the simplicity, low cost and rapidity of such an assay system.

Significant variability in both plaque and saliva urease levels was observed between the subjects in each group, and also between the two samples taken from the same subject on different days. We feel confident that this variability is not the result of a methodological error, because the assay used is highly reproducible. In fact, three identical assays are always performed simultaneously from each sample, and those always demonstrate minimal variation (estimated intraclass correlation $\rho_I = 0.654$, 95% CI 0.499, 0.808). The observed variability in urease levels both among and within subjects can be due to differences in the microbial composition of the plaque and saliva samples. In other words, it is possible that highly ureolytic plaque or saliva samples may contain a higher proportion of bacterial species that are capable of alkali generation via the urease pathway. Alternatively, the observed differences in urease levels between samples could be attributed to differences in the level of expression of the urease genes brought on by differences in environmental conditions in the plaque samples. We have previously shown that urease expression in oral bacteria can be regulated by induction by low pH and high carbohydrate concentrations, or by nitrogen limitation (7, 21). It is therefore possible that the observed variability of the urease levels reflects a more complex interaction between diet, pH, urease gene expression, ammonia production and changes in microbial composition. To answer these questions, we are presently adapting real-time polymerase chain reactions using primers from conserved regions of the urease genes against RNA and DNA samples obtained from human plaque to

quantify the numbers of ureolytic bacteria as well as the expression of urease genes in human plaque *in vivo*. Other methods to try to enumerate ureolytic organisms in human plaque samples have not yielded definitive results, apparently as a result of the instability of the urease phenotype associated with even short-term *in vitro* passage of ureolytic oral isolates (28).

Impressive sensitivity, specificity and ROC results were obtained in the present study when different plaque urease levels were used as cut-off points to correctly assign the plaque urease measurements to CA and CF subjects. However, this could be the result of the significant difference in the caries status of the two groups (no caries vs. six open lesions). This analysis indicated that only 7% of the plaque urease measurements from CA subjects were > 2.2 units/mg, (where 1 unit is defined as 1 μmol urea hydrolysed/min/mg protein) while only 8% of the plaque urease measurements from CF subjects where lower than 0.9 units/mg. A similar pattern was not observed for saliva urease because 33% of the salivary urease measurements from CA subjects were in the highest quintile compared to only 16% of the measurements from CF subjects (data not shown). Based on these observations, one may speculate that plaque urease levels <1 unit/mg protein are mostly associated with increased caries levels, while plaque urease levels above 2 units/mg protein, are mostly associated with dental health. A larger prospective cohort study will be required to determine what levels of urease are minimally required to confer protection against dental caries. In addition, such a study could also provide the opportunity to evaluate the predictive power of plaque urease activity levels in assessing the risk of an individual to develop caries in the future.

In summary, this study provides clinical evidence, in contrast to a previous report (13), that loss of alkali-generating capacity in dental plaque from urea hydrolysis may be associated with the development of dental caries. Further prospective studies are needed to determine whether the association between low plaque urease activity and increased caries levels observed in the present study is in fact a cause–effect relationship. Further studies are also needed to evaluate the stability of urease levels over time before we can explore the utility of this relationship in the development of novel approaches for caries risk assessment. In addition, strategies that target the enhancement or stabilization of ammonia production in plaque could prove to be highly effective in controlling caries according to the results of this study.

Acknowledgments

We thank Dr Susan Nimmo of the Department of Operative Dentistry, University of Florida for selecting subjects with active caries and the staff of the University of Florida Periodontal Disease Research Center, Lorraine Benz, Brenda Hales and Shari Brandemuhl, for selecting caries-free subjects and offering clinical support for the collection of plaque and saliva samples. We would also like to thank Drs Ronald Billings and Dorota Kopeycka-Kedzierawski from the University of Rochester, Dr Augusto Elias from the University of Puerto Rico and Dr Walter Psotter, New York University and the University of Puerto Rico for their critical evaluation of the manuscript. This study was supported by National Institute of Dental Research Grant RO1 DE10362 (PI: Robert A. Burne) with additional support by RCRII Grant 1P20RR11126 from the National Center for Research Resources.

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