The Association of Bacterial Adhesion with Dental Caries

INTRODUCTION

Dental caries is an infectious disease with life-style and genetic factors modifying disease activity (Stamm et al., 1993; Fejerskov, 1997). Today, only a small portion of Western populations display high caries activity (Flinck et al., 1999), highlighting the possibility of genetic factors in caries development (Conry et al., 1993).

Saliva contains a multitude of factors important to oral microbial ecology, biofilm formation, and health (Gibbons, 1989; Lamkin and Oppenheim, 1993; Whittaker et al., 1996). Thus, loss of salivation may result in rampant tooth decay. However, attempts to correlate salivary factors with caries have been conflicting. Indeed, some studies, though not others, have claimed streptococcal adhesion (Rosan et al., 1982), agglutinin (Tenovuo, 1997), acidic PRPs (Yu et al., 1986), specifically Db (Friedman et al., 1980), and basic proline-rich peptides (Ayad et al., 2000), to play a role in dental caries.

Adhesion of bacteria by salivary components is a key event in oral biofilm formation (Gibbons, 1989). Acidic PRPs are multifunctional proteins (Lamkin and Oppenheim, 1993; Hay et al., 1994) mediating avid binding to hydroxyapatite by commensal Actinomyces and Streptococcus species (Gibbons et al., 1991; Li et al., 1999), as well as low-avidity binding by S. mutans (Gibbons et al., 1991). Acidic PRPs, encoded by the PRH1 and PRH2 gene loci, contain allelic variants, derived by amino acid substitutions (PRP-1, PRP-2, PRP-3, and Db-1) (Azen, 1993; Hay et al., 1994).

Salivary agglutinin is an innate immunity scavenger receptor cysteine-rich gp-340 protein (Prakorbphol et al., 2000) attaching S. mutans to hydroxyapatite surfaces (Gibbons, 1989; Carlén et al., 1998). While acidic PRPs promote adhesion on surfaces only (Gibbons et al., 1990), agglutinin is active in saliva (i.e., aggregation). Consequently, agglutinin is considered a clearance factor of S. mutans.

Partial least-squares (PLS), a statistical method originally designed for multivariate problems in chemometrics, has until now not been applied in the correlation of saliva variables with caries (Wold et al., 1998). PLS lacks some of the limitations of other multivariate methods, since it (i) handles multiple collinear variables by far exceeding the number of observations (or subjects) and (ii) utilizes all information of the variable set without schemes for reducing the variables down to a few or using cut-off values.

The present study aims at using PLS to correlate bacterial adhesion and related salivary ligands with high (n = 19) and low (n = 19) caries experiences in 38 children, selected from 3400 12-year-old Swedish children with today’s skewed caries distribution. We found adhesion of cariogenic and commensal model bacteria to correlate with susceptibility and resistance to caries, respectively.

ABSTRACT

Saliva adhesion of bacteria is a key event in oral biofilm formation. Here, we used partial least-squares (PLS) analysis to correlate adhesion of cariogenic (Streptococcus mutans Ingbritt) and commensal (Actinomyces naeslundii LY7) model bacteria, and their agglutinin and acidic proline-rich protein ligands, respectively, with high and low caries experiences in 38 children reflecting today’s skewed caries distribution. Adhesion of S. mutans was among the factors correlating strongest with high caries experience when PLS modeled together with traditional factors (e.g., sugar intake, lactobacilli counts). Saliva phenotypes with high agglutinin levels and Db-s (an acidic PRP variant) coincided with both high caries experience and S. mutans adhesion. A. naeslundii adhesion correlated with low caries experience. Non-Db phenotypes (i.e., acidic PRP-1 and PRP-2 variants) coincided with both low caries experience and S. mutans, but high A. naeslundii adhesion. Thus, bacterial adhesion may modulate susceptibility and resistance to dental caries.

KEY WORDS: acidic proline-rich proteins, agglutinins, bacterial adhesion, dental caries susceptibility.
Baseline caries experience (totally, 207 lesions of 4414 scored surfaces) was recorded in 1995, and new or progressing enamel and dentin lesions (n = 132) at a two-year follow-up (1997), within the framework of the nationwide cohort study (Källestål et al., 2000a). All enamel caries (n = 112) occurred at smooth (buccal, lingual, and proximal) surfaces, and most of enamel/dentin caries (n = 39) and fillings (n = 56) at occlusal surfaces (n = 24 and 41, respectively). No teeth were missing due to caries. Baseline data on sugar intake, oral hygiene, and fluoride exposure were obtained by means of a validated questionnaire (Flinck et al., 1999; Källestål et al., 2000a).

**Saliva Collection**

Parotid saliva, stimulated by 3% citric acid, was collected on ice by means of Lashley cups at the same hour of the day at the dental clinics (June, 1996, and April-September, 1997). The saliva samples (up to 4 mL per subject) were aliquoted and stored frozen (80°C) prior to use. Whole saliva, stimulated by the subjects' chewing on paraffin, was collected on ice prior to analyses of flow rate, pH, and buffer capacity, and counts of S. mutans, total streptococci, and lactobacilli.

**Bacterial Adhesion**

Adhesion of model bacteria [d. naeslundii strains LY7 (Carlén et al., 1998; Strömberg et al., 1992) and P-4-N (Hallberg et al., 1998) and S. mutans strain Ingbrit (Carlén et al., 1998)] to hydroxyapatite beads coated with parotid saliva was done as described (Gibbons et al., 1991; Carlén et al., 1998).

The saliva concentrations mediating dose-dependent adhesion by each model bacterium was established by the use of serial dilutions of parotid saliva from three subjects. All 38 saliva samples were then tested at 1 or 2 saliva dilutions ([1:1 for P-4-N, 1:1 and 1:10 for Ingbrit, and 1:64 and 1:128 for LY7]) within the dose-dependent adhesion intervals. Adhesion of strain LY7 by saliva collected in 1996 and 1997 displayed an intra-subject correlation coefficient of 0.54 for all samples. Other salivary analyses were not repeated in 1997.

**Acidic Proline-rich Proteins**

Acidic PRPs were separated by native alkaline electrophoresis in 4 to 7% polyacrylamide gels as described (Azén and Yu, 1984). PRP-1 and PIF was distinguished by isoelectric focusing (Azén and Denniston, 1981). The gels were scanned (Model GS-700 Imaging Densitometer, Bio-Rad, Hercules, CA, USA), and acidic PRPs were quantified by means of the Molecular Analyst® software (Bio-Rad). Total acidic PRPs were quantified by fast protein liquid chromatography (FPLC, Biologic®, Bio-Rad) and the software Peak Fit™ (Jandel Scientific GmbH, Erkrath, Germany).

**Salivary Agglutinin**

Agglutinin was quantified by means of a slot-blot assay. Two saliva dilutions (1:50 and 1:100), selected within the dose-dependent antibody-binding interval, were transferred to PVDF membranes (Immobilon 0.45 mm, Millipore, Bedford, MA, USA). After being blocked with 5% dry milk in Tris-buffered saline (TBS, 0.02 M Tris/Tris-HCl, 0.5M NaCl, pH 7.5) with 0.05% Tween-20 (TTBS), the membranes were washed and incubated for 1 hr with agglutinin-specific mAb143 (1:60,000, Dr. Malamud, University of Pennsylvania, Philadelphia; Takano et al., 1991). After washes, the membranes were incubated for 1 hr with HRP-conjugated rabbit anti-mouse IgG antibodies (1:2000, DAKO A/S, Glostrup, Denmark). After washes, binding was visualized (SuperSignal® Substrate, Pierce, Rockford, IL, USA). The films were then scanned, and agglutinin was quantified by densitometry.

**MATERIALS & METHODS**

**Study Group and Clinical Recordings**

Nineteen high-caries cases and 19 low-caries referents (12 years of age), from three Public Dental Health Clinics, participated. The 38 children (20 boys, 18 girls) were nested in the northern portion of a Swedish nationwide cohort of 3400 children (Flinck et al., 1999; Källestål et al., 2000a). The cases (mean baseline DMFS = 5.0, two-year DMFS increment = 2.4) and referents (baseline DMFS = 0, mean two-year DMFS increment = 0.6), respectively, were randomly selected from among the children displaying 4 or more new lesions (enamel caries included) during the latest year, and from among caries-free subjects living in the same area. The study was approved by the Ethics Committee at Umeå University, Umeå, Sweden.
Partial Least-squares (PLS)

PLS was performed as described (Wold et al., 1998). In principle, PLS establishes the correlation structure between one data matrix of descriptor (X) and another of response (Y) variables, in a multivariate model. We established the correlation structure between 3 X and 5 Y matrices with single or multiple individual y variables (models I-V, Appendix, www.dentalresearch.org); one X-matrix (saliva adhesion, acid PRPs, and agglutinin, and traditional microbial, salivary, and life-style x variables) was correlated to multiple (model I) or singular y carries variables (DMFS, model II), and a second X-matrix (the same x variables combined with baseline carries) to two-year carries increment (model III), and a third X-matrix (acidic PRPs and agglutinin x variables) to A. naeslundii (model IV) and S. mutans (model V) adhesion.

Univariate Statistics

The differences between means for high- and low-caries groups and different PRP phenotypes were tested by multiple mean testing (Tukey's test). The proportion of Db-positive children in the high- vs. low-caries groups was tested by Ch² test. A P-value below 0.05 was used to indicate a statistically significant difference between groups. One-sided testing was accepted, since univariate testing was applied only for influential (VIP > 1.0) variables in the PLS modeling. In addition, the odds ratio for the Db phenotype in the high- compared with the low-caries group was analyzed by multivariate logistic regression (Egret, Seattle, WA, USA).

RESULTS

Bacterial Adhesion Coincides with Caries Experience

We used PLS to correlate saliva adhesion of S. mutans Ingbrit and A. naeslundii LY7 with high (n = 19) and low (n = 19) carries experiences in 12-year-old children (Table 1). The entire variable set, traditional variables included (Table 1), explained (R²) and predicted (Q²) a significant portion of the variance in carries experience (R² = 0.388, Q² = 0.182, PLS model I).

A comparably high adhesion of S. mutans (binding to agglutinin) and A. naeslundii (binding to acid PRPs) correlated with high and low carries experiences (VIPS = 1.79 and 1.40, respectively (Table 1). In contrast, adhesion of A. naeslundii P-4-N (binding to β-linked galactose) did not correlate with carries experience. Univariate statistics verified that saliva from high-caries subjects bound more S. mutans cells than saliva from the low-caries subjects (p = 0.049), while the opposite was true for A. naeslundii cells (p = 0.011) (Table 4).

Highly influential PLS correlations (VIP > 1.5) with baseline carries experience occurred for S. mutans adhesion and some traditional factors, e.g., fluoride use (VIP = 1.69) and lactobacilli counts (VIP = 1.70), while others, e.g., sugar intake, pH, and oral hygiene, were less influential (VIP = 1 < 1.5) (Table 1). In addition, S. mutans adhesion, though not lactobacilli counts, displayed influential PLS correlations when the variable set was analyzed against two-year carries increment (Table 2).

Salivary Ligands for Bacterial Adhesion Coincide with Caries Experience

We analyzed the same variable set by PLS to correlate the salivary ligands for S. mutans (agglutinin) and A. naeslundii (acidic PRPs) with high and low carries experiences (Table 1). The variant PRP types, measured by electrophoresis, behaved differently relative to carries experience (Table 1). While Db-s coincided with high carries experience (VIP = 1.13), the non-Db types (PRP-1/PF-s, PRP-2, and Pa) coincided with low carries experience (VIP = 1.00). Univariate comparisons verified Db⁺ and Db⁻ phenotypes to differ in carries experience (p < 0.05), and the Db⁻ phenotypes were nearly twice as common among high- (39%) compared with low- (21%) carries subjects (Table 4).

A comparably high amount of total acidic PRPs, measured by FPLC, coincided with low carries experience (VIP = 1.46), while high levels of agglutinin, measured by immunodetection, displayed a tendency to coincide with high carries experience (VIP = 0.93) (Table 1). In addition, the small acidic PRPs (e.g., PRP-3 and PRP-4) behaved similarly to their large counterparts (e.g., PRP-1 and PRP-2).

Agglutinin and Acidic PRPs Coincide with Bacterial Adhesion

The PLS correlations showed the presence of Db-s (VIP = 1.08) and high amounts of agglutinin (VIP = 1.85) to coincide with high adhesion of S. mutans (Table 3). Univariate statistics verified that Db⁺ saliva bound more S. mutans cells than did Db⁻ saliva (p = 0.06) (Table 4). Neither Db nor agglutinin coincided with adhesion of A. naeslundii (Table 3).

The presence and high amounts of non-Db types (PRP-1/PF-s, PRP-2, and Pa) correlated with high A. naeslundii (VIP = 1.09), but low S. mutans (VIP = 1.35), adhesion (Table 3). PRP-2, PRP-4, and Pa each correlated with high A. naeslundii, but low S. mutans, adhesion, while PRP-1/PF-s did not. Univariate statistics verified that PRP-2⁺ saliva bound more A. naeslundii cells than did PRP-2⁻ saliva (p = 0.003), while the opposite was true for S. mutans (p = 0.044) (Table 4).
### Table 3. PLS Associations of Acidic PRPs and Agglutinin with Adhesion of Commensal (A. naeslundii) and Cariogenic (S. mutans) Model Bacteria

<table>
<thead>
<tr>
<th>x Variables</th>
<th>A. naeslundii LY7 Adhesion</th>
<th>S. mutans Ingbritt Adhesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRP-2</td>
<td>1.18 + Db-s</td>
<td>1.08 +</td>
</tr>
<tr>
<td>PRP-4</td>
<td>1.16 + PIF f</td>
<td>0.40 +</td>
</tr>
<tr>
<td>Pa</td>
<td>1.14 + PRP-1/PIF-s</td>
<td>0.15 +</td>
</tr>
<tr>
<td>Non-Db types</td>
<td>1.09 + PRP-3/PIF-f</td>
<td>0.05 +</td>
</tr>
<tr>
<td>PRP-2, PRP-1/PIF-s</td>
<td>0.68 + PRP-4</td>
<td>1.43 +</td>
</tr>
<tr>
<td>PRP-3/PIF-f</td>
<td>0.71 + Pa</td>
<td>1.18 +</td>
</tr>
<tr>
<td>Db-s</td>
<td>0.40 — PRP-2</td>
<td>1.11 +</td>
</tr>
<tr>
<td>PRP-total</td>
<td>1.88 + PRP-total</td>
<td>0.32 +</td>
</tr>
<tr>
<td>Agglutinin total</td>
<td>0.81 + Agglutinin total</td>
<td>1.85 +</td>
</tr>
</tbody>
</table>

* PLS model IV (Y = Actinomyces adhesion; R² = 0.576, Q² = 0.424). The Y matrix is composed of 2 y variables reflecting adhesion of A. naeslundii LY7 to saliva-coated hydroxyapatite beads (www.dentairesearch.org).
* PLS model V (Y = S. mutans adhesion; R² = 0.250, Q² = 0.088).
* Both arbitrary concentrations (units/mL) and outputs (units secreted/min) of the variables were included in the X matrices. VIP values for caries lesions are given, but variable concentration and output had virtually identical VIP values.
* VIP ≥ 1.5 marks highly influential and VIP = 1.0-1.5 influential variables.
* Positive (+) or negative (-) direction of the correlation.
* Non-Db types = PRP-1/PIF-s, PRP-2, and Pa.
* PIF identified by isoelectric focusing and scored as present (yes) or absent.

### Table 4. Univariate Comparisons of High- and Low-caries Groups and PRP Phenotypes

<table>
<thead>
<tr>
<th>Variable</th>
<th>High-caries (n = 19)</th>
<th>Low-caries (n = 19)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. mutans adhesion</td>
<td>29.4 ± 4.9</td>
<td>19.1 ± 3.7</td>
<td>0.049</td>
</tr>
<tr>
<td>A. naeslundii adhesion</td>
<td>47.5 ± 1.4</td>
<td>53.0 ± 1.5</td>
<td>0.011</td>
</tr>
<tr>
<td>Db phenotype prevalence (%)</td>
<td>39</td>
<td>21</td>
<td>0.120</td>
</tr>
<tr>
<td>Odds ratio (95% CI)</td>
<td>4.7 (0.8-28.3)</td>
<td>1.0</td>
<td>0.045</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variable</th>
<th>Db-positive (n = 11)</th>
<th>Db-negative (n = 26)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline caries (DMFS)</td>
<td>4.2 ± 1.4</td>
<td>1.8 ± 0.6</td>
</tr>
<tr>
<td>S. mutans adhesion</td>
<td>31.8 ± 5.6</td>
<td>21.0 ± 3.6</td>
</tr>
<tr>
<td>A. naeslundii adhesion</td>
<td>51.2 ± 1.5</td>
<td>49.9 ± 1.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>PRP-2-positive (n = 20)</th>
<th>PRP-2-negative (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. mutans adhesion</td>
<td>19.2 ± 3.5</td>
<td>29.9 ± 8.5</td>
</tr>
<tr>
<td>A. naeslundii adhesion</td>
<td>53.2 ± 1.5</td>
<td>47.7 ± 0.11</td>
</tr>
</tbody>
</table>

* Thirty-eight 12-year-old children classified as high-caries cases (mean baseline DMFS = 5.0) and low-caries referents (baseline DMFS = 0). These children were stratified by PRP phenotype (i) Db variant present (positive) or absent (negative) or (ii) PRP-2 variant present (positive) or absent (negative).
* p-values from one-sided hypothesis testing by Tukey’s multiple means test or Chi² test, or multiple logistic regression, including sugar intake and oral hygiene as covariates.
* Mean ± SE of number of caries lesions or percent bound bacteria. Adhesion of S. mutans Ingbritt and A. naeslundii LY7 (saliva dilutions 1:1 and 1:128, respectively) to parotid saliva coated onto hydroxyapatite beads.

### DISCUSSION

This study suggests bacterial adhesion as a determinant of susceptibility and resistance to dental caries. The 38 test subjects, selected from a nationwide cohort of 3400 12-year-old Swedish children with today’s skewed caries distribution, displayed odds ratios for traditional life-style factors similar to those of the entire cohort. The traditional factors (e.g., sugar intake, toothbrushing) poorly explained the variance in caries in the entire cohort (Finck et al., 1999), while self-esteem coincided with oral health behavior and caries experience (Källesjö et al., 2000b). Thus, the present study may indicate a role of genetic factors in caries development and today’s skewed caries distribution in Western populations (Stamm et al., 1993; Fejerskov, 1997).

The present correlation of S. mutans and A. naeslundii adhesion with high and low caries experiences, respectively, suggests an inverse relationship of adhesion of cariogenic and commensal bacteria to caries. Adhesion of S. mutans ranked comparably high when PLS modeled among traditional factors (e.g., sugar intake, lactobacilli counts), a finding that holds true for both caries experience and two-year increment. Moreover, the salivary ligands for S. mutans (agglutinin) and A. naeslundii (acidic PRPs) correlated with both caries experience and adhesion. Consequently, adhesion and host polymorphism may influence susceptibility and resistance to caries by affecting oral microbial ecology and innate immunity.

The present findings suggest that the acidic PRP phenotype (content of Db and non-Db types) may modulate both caries experience and adhesion. The Db variant, characterized by a 21-amino-acid internal tandem repeat, coincided with both high caries experience and S. mutans adhesion. The Db-type, earlier correlated with caries experience (Friedman et al., 1980), was almost twice as common among high- (39%) compared with low- (21%) caries subjects. Mechanistically, Db may hold a unique conformation, attaching S. mutans directly (Gibbons, 1989) or by affixing agglutinin ligands onto the hydroxyapatite surface. In contrast, the non-Db types (PRP-1/PIF-s, PRP-2, and Pa) coincided with both low caries experience and S. mutans, but high A. naeslundii, adhesion. Thus, non-Db types may provide A. naeslundii, while masking S. mutans, adhesion sites. Finally, Db and non-Db types may also differ in other innate immunity properties, e.g., acidic PRP proteolysis to innate immunity peptides (Li et al., 2000).
The present findings showed high levels of agglutinin to coincide strongly with S. mutans adhesion, but weakly with caries experience. The weak correlation of agglutinin with caries may relate to its aggregation (clearance) of S. mutans in saliva, while the more direct relationship of Db/non-Db types with adhesion and caries experience may relate to the cryptic behavior of acidic PRPs in saliva. Nevertheless, since agglutinin and Db explained only a portion of the variance in S. mutans adhesion, yet other molecules may also account for the individual saliva adhesion capacity of S. mutans (Carlén et al., 1998).

The present study illustrates the power of PLS to correlate multiple saliva variables with caries. Although the univariate comparisons displayed similar associations and trends, PLS identifies (i) the relative importance of individual variables and (ii) the explanatory and predictive capacity of the entire variable set. Thus, PLS may be ideally suited to reveal disease-associated variable patterns, as well as those patterns suited for diagnostic purposes. Anyhow, this study utilized only a limited number of test subjects, and further studies are required to explore the role of saliva polymorphism and PLS fingerprinting in diagnostic and preventive strategies to control dental caries. Finally, studies along this line may also generate therapeutic agents to control oral biofilm formation and dental diseases.

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