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ATP Bioluminescence: Rapid Quantification of Oral Bacteria

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ABSTRACT

Dentistry has undergone a shift in caries management towards prevention and improved diagnosis. This cross-sectional study demonstrates the use of ATP bioluminescence as an innovative tool for the rapid chair-side enumeration of oral bacteria. Thirty pediatric patients were examined, and plaque specimens, in addition to stimulated saliva, were collected at representative teeth within each quadrant. Oral specimens (n = 150) were assessed by plating on enriched and selective agars, and also subjected to ATP bioluminescence determinations using a luciferase-based assay system. Statistical correlations, linking ATP values to numbers of total bacteria, oral streptococci and mutans streptococci, yielded highly significant *r* values of 0.854, 0.840, and 0.796, respectively. Our clinical data is consistent with the hypothesis that ATP measurements have a strong statistical association with bacterial number in plaque and saliva specimens, including numbers for cariogenic mutans streptococci, and may be used as a potential assessment tool for dental caries risk.

INTRODUCTION

Dental caries is a microbial disease where the principal cariogenic microorganisms have been identified as mutans streptococci and lactobacilli (Zero, Fontana and Lennon, 2001; Saini *et al.*, 2003; Bowden *et al.*, 1990; Kohler, Andreen, and Jonsson, 1988). These bacteria are normal constituents of the oral microflora, and are typically transmitted from mother (or primary caretaker) to child within a several year period following tooth eruption (Law, Seow, and Townsend, 2007; Li *et al.*, 2005)). When large numbers of these cariogenic bacteria adhere to the tooth surface in the form of plaque biofilm, ingested sugars are converted by glycolysis to weak organic acids that attack the tooth surface by demineralization of the hydroxyapatite structure. Between periods of acid generation due to food ingestion, buffering agents in saliva and plaque neutralize the organic acids and arrest the demineralization process. Remineralization of tooth hydroxyapatite occurs between periods of demineralization; thus a dynamic flow of calcium and phosphate into and out of the tooth enamel takes place. If demineralization occurs too frequently, it eventually causes permanent destruction of the tooth structure with the development of white spot lesions and the formation of cavities (Zero, Fontana and Lennon, 2001).

There is a critical need in dentistry to develop better assessment methods to determine patient risk for dental caries, since both disease as well as treatment result in the irreversible loss of tooth structure. Reliable caries risk assessment would identify high-risk patients, and allow targeting of more aggressive, caries-protective treatments.

Caries risk assessment tools are an important diagnostic aid in the field of pediatric dentistry. The etiology of caries is multi-factorial, and caries-risk predictors may be found among the oral microflora, the diet, and the host, all three of which are essential for caries development (Keyes and Jordan, 1963). Previous caries risk indexes have been directed at the evaluation of social, behavioral, microbiologic, environmental, and clinical variables. Many of these variables, such as frequency of dental visits, socioeconomic status, exposure to sugars, fluoride exposure, brushing habits, and visible plaque, are primarily subjective observations based on accounts in health history. As a means of increasing the quantitative power of caries risk assessment tools, the American Academy of Pediatric Dentistry (AAPD) has recently promoted the use of microbiological testing as an adjunct measure for assessing dental caries risk (AAPD, 2003 and 2006).

Rapid ATP (adenosine triphosphate) bioluminescence assays have long been used as a quantitative measure of microbial numbers, and more recently in dental plaque (Ronner *et al.*, 1999). Bioluminescence assays measuring energy metabolites, including ATP, have been identified to retain high correlations with plaque mass obtained from both humans and animal subjects (Ronner *et al.*, 1999).

The purpose of this study is to demonstrate the use of ATP bioluminescence as an innovative tool for the rapid chair-side enumeration of total oral bacteria, including cariogenic bacteria. Using plaque and saliva specimens from 30 participants in a cross-sectional study, we compared ATP-driven bioluminescence derived from oral specimens to bacterial number quantitated using standard microbiological plating methods. This study tested the hypothesis that ATP measurements obtained from oral clinical specimens can be used as a direct assessment of cariogenic bacterial numbers and by

extrapolation dental caries risk. In this study, we also developed a quantitative caries risk assessment tool that combined clinical and microbiological evaluations.

MATERIALS AND METHODS

Patients Enrolled in the Clinical Study

Patients registered for dental care in the OHSU Pediatric Dentistry Clinic were randomly selected for inclusion in this study. The criteria used for selection was age (7-12 years), demonstrated good health and the ability to expel saliva. The criteria for exclusion in this study were the wearing of oral appliances because of its demonstrated ability to modify surface characteristics, and any saliva and/or diet altering medications. Consent forms for routine dental care and for the research study were presented to the participant and parent/guardian. All human subject protocols, use of consent forms, and specimen collections were reviewed and approved by the OHSU Institutional Review Board. The oral examination included indication of missing, decayed and restored teeth (Table 1), as well as other information such as partially erupted teeth and restorative materials.

Plaque Specimens Collected From Specific Teeth

Four teeth representing all quadrants of the mouth were tested in each patient (Table 1). Teeth such as the upper right first molar and lower left molar were chosen because of their difficulty for brushing in most patients, and also because of the opposing proximity, near or far distance, to a salivary duct for each tooth. An upper incisor was chosen because of its susceptibility to show enamel demineralization and significant plaque accumulation in children (Goodman and Armelagos, 1985; Dummer *et al.*, 1986; Li *et al.*, 1995). Finally, a lower incisor was chosen because of its close proximity to a salivary gland and the enhanced cleansing properties of the tongue.

Microbiological Identification of Plaque and Saliva Bacteria

Each plaque specimen was weighed, then suspended in 1 ml of phosphate buffered saline (PBS) with the addition of glass beads, and dispersed by vigorous agitation. Dispersed plaque, as well as saliva, were subjected to 10-fold serial dilutions in PBS and then plated on enriched blood agar (PML Microbiologicals, Wilsonville, OR) to determine total bacterial numbers. Total oral streptococci were determined by limiting dilution plating on Mitis Salivarius agar (MS agar; Difco™; Becton, Dickinson and Company, Sparks, MD) supplemented with potassium tellurite (Difco™). For selection of mutans streptococci, MS agar including potassium tellurite, was supplemented with bacitracin (10 Units/ml; Sigma Chemical, St. Louis, MO). All platings were conducted in quadruplicate, and plates exhibiting colony numbers between 50-500 were counted and averaged. As an additional validation in the determination of dental caries risk, we used the CRT Bacteria Test (Vivadent, Lichtenstein, Germany), which also incorporates MS agar and Rogosa agar in a dual slide test.

ATP Bioluminescence Determinations

ATP contained in bacteria from plaque or saliva specimens was determined with the use of the BacTiter Glo Microbial Cell Viability kit (Promega, Madison, WI), with ATP-driven bioluminescence measured by the Veritas Microplate luminometer (Turner Biosystems, Inc., Sunnyvale, CA). Relative light units (RLUs) were calibrated using a standard curve of ATP (pM or higher; Sigma Chemical) and correlated against bacterial cell number. In parallel bioluminescence assays, we utilized the CariScreen ATP bioluminescence swab collection system and hand-held ATP Meter (Oral Biotech, Albany, OR).

RESULTS AND DISCUSSION

ATP Standards in Bioluminescence Measurements

Using ATP standards in picomolar (pM) or nanomolar (nM) concentrations, bioluminescence (RLUs) standard curves were developed for both the Veritas luminometer and CariScreen ATP Meter (Figure 1A, left and right panels, respectively). The Veritas luminometer has greater than 5 logs dynamic range measuring ATP from 10 pM to 1 μM (with RLU readouts from 10^4 - 10^8 RLUs), and the CariScreen meter has approximately 100-fold dynamic range measuring ATP from 100 pM – 10 nM (with RLU readouts from 15 – 4000 RLUs). Thus, plaque and saliva specimens were subjected to 10-fold serial dilutions, to insure that at least one of the bioluminescence readouts was measured in the linear portion of the dynamic range for each luminometer. This process was conducted to insure that the most accurate RLU value, which would measure ATP concentration most reflective of true cell number, could be compared to bacterial cell numbers enumerated directly by plating on enriched or selective agars.

Statistical Correlations Linking ATP Bioluminescence to Total Oral Bacteria and Oral Streptococci.

Saliva and plaque specimens were collected and ATP-driven bioluminescence was measured from each specimen. Using serial dilution plating of each specimen, quantitation was conducted for total bacteria on enriched medium (blood agar) and total streptococci and mutans streptococci on selective medium (MS agar), in the absence or presence of bactracin, respectively. When ATP bioluminescence values, using the Veritas luminometer, were determined using our composite plaque and saliva data set, strongly-significant Pearson correlation coefficients of 0.854, 0.840, and 0.796 were

determined for total oral bacteria, total oral streptococci, and mutans streptococci, respectively (with 1.0 being a perfect correlation; Figure 1B). When these ATP bioluminescence readings were analyzed using plaque specimens only, which in this case reduces the statistical power because of a smaller sample set, significant correlation coefficients of 0.682, 0.611, and 0.548 were still identified for total oral bacteria, total oral streptococci, and mutans streptococci, respectively (Figure 1C). When scatter plot analyses were conducted correlating total oral bacteria with either total oral streptococci or mutans streptococci (Figure 1D), increasing numbers of total oral bacteria were found to track linearly with total oral streptococci in a strongly significant relationship (r = 0.942), and also to a lesser but still significant degree with mutans streptococci (r = 0.700).

Similar ATP bioluminescence relationships were found using the handheld CariScreen ATP Meter, where bioluminescence readouts for composite plaque and saliva specimens, or for plaque specimens alone, correlated well with numbers determined for total oral bacteria, total oral streptococci, and mutans streptococci (Figure 2A and 2B; r values of 0.810, 0.780 and 0.753, respectively, using composite plaque and saliva specimens, and r values of 0.587, 0.509, and 0.471, respectively, using plaque specimens only).

Thus, ATP-driven bioluminescence is highly predictive of the numbers of total oral bacteria and total oral streptococci, and by statistical extension, is also reflective of the numbers of cariogenic mutans streptococci in oral specimens. Strong statistical correlations were determined using either the Veritas luminometer or the CariScreen ATP Meter, but only when using the increased statistical power of the larger sample number contained in the composite plaque and saliva specimen set.

Criteria Used for Development of OHSU Pediatric Dentistry Caries Risk Index (CRI)

In the patient population at the OHSU Pediatric Dentristry Clinic, all children would be considered high risk according to the currently recommended AAPD Caries Assessment Tool. Furthermore, the AAPD Caries Assessment Tool holds previous caries-related history as a bias against potential reassessment of future caries risk. In order to be able to quantify and rank our study population, a composite of quantifiable clinical and microbiological evaluation criteria was included in our CRI score.

Three index factors were used to develop the OHSU Pediatric Dentistry caries risk index. The first factor was visible bacterial plaque without the use of disclosing solution. Alaluusua (1994) demonstrated that visible plaque was strongly associated with caries development. Wendt *et al.* (1996) also determined that children with no visible plaque at two years of age had greater chances of remaining caries-free until three years of age, compared to children with visible plaque. While in some studies, supragingival plaque has not been highly correlated with caries experience (Frans and Baume, 1983; McHugh, 1986), studies by Lindhe *et al.* (1975) and Poulsen *et al.* (1976) demonstrated that professional plaque removal could prevent caries (Leverett *et al.*, 1993).

The second index factor was presence of active caries. Zero, Fontana and Lennon (2001) stated that the best predictor for caries in primary teeth was previous caries experience. Li and Wang (2002) demonstrated that caries in the primary dentition was predictive of caries in the permanent dentition. To illustrate the continuum of cariogenic disease, Greenwell *et al.* (1990) found that 84% of the children who did not display caries in the primary dentition remained caries-free in the mixed dentition.

The third index factor was levels of mutans streptococci and lactobacilli. Individuals with active caries tend to harbor higher numbers of mutans streptococci and lactobacilli than individuals who are caries-free (Leverett, 1993; Alaluusua *et al.*, 1987; Krasse, 1988; Loesche, 1988). In longitudinal studies, increased numbers of mutans streptococci and lactobacilli were associated with the onset and progression of caries (Loesche *et al.*, 1984; Boyar and Bowden, 1985; Lang *et al.*, 1987; Kingman *et al.*, 1988). Children at infancy with high levels of mutans streptococci, compared to older children, were found to possess more severe caries in the primary dentition (Alaluusua and Malmivirta, 1994; Mundorff *et al.*, 1993; Anderson and Shi, 2006).

Quantitative Formulation of OHSU Pediatric Dentistry Caries Risk Index

Thus, the OHSU Pediatric Dentistry CRI score is composed of measures evaluating traditional plaque index, number of cavitated surfaces, and CRT results enumerating numbers of mutans streptococci and lactobacilli (weighted 25%, 25% and 50%, respectively; Figure 3A and 3B). Values from all categories were then added to develop the composite CRI score, and designated as low, moderate, or high caries risk. The ranges of composite scores for determination of low, moderate and high caries risk were 4-6, 7-9 and 10-16, respectively (Figure 3B). By combining these three risk factors, the goal was to eliminate qualitative personal observations, and develop a scientific-based quantitative assessment tool using indicators that were more reliant on current patient status, for direct comparison to measurements of ATP bioluminescence identified in this study.

Relationship Between ATP Bioluminescence and Caries Risk

There appears to be a trend for stepwise increase in ATP bioluminescence obtained from the saliva specimen for any given patient, when plotted against the broad categories of patients exhibiting low and moderate / high caries risk (Figure 3C). Due to the almost exclusive number of high caries risk individuals seen at the OHSU Pediatric Dentistry Clinic, the sample size for low caries risk individuals was determined to be insufficient to make definitive comparisons. Based on ATP bioluminescence values alone, we are unable to segregate individuals between the moderate and high caries risk categories, and consider patients to be either in the category of low caries risk or the broad category of moderate / high caries risk.

This study has provided validation that ATP-driven bioluminescence may be used as a potential quantitative biomarker of total oral bacteria that could be rapidly and reliably measured at the chair-side. The strong statistical correlation between total oral bacterial number to numbers of oral streptococci and cariogenic mutans streptococci infers that ATP bioluminescence may potentially serve as a quantitative assessment determinant of dental caries risk. This work has broad implications in dentistry and medicine, and can be used translationally in the clinic to determine the efficacy of interventional therapies for dental caries, and perhaps for detection of bacterial infections in periodontal and other infectious diseases.

ACKNOWLEDGEMENTS

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Table 1: Patient Demographics and Clinical Observations[1]

Gender		Age		Ethnicity		Residence		Medical History[2]		Additional Fluoride Use[3]	
	Number of Patients		Number of Patients		Number of Patients		Number of Patients		Number of Patients		Number of Patients
Male	23	7 yrs	9	African-American	3	City of Portland	12	ASA 1	29	In Water	7
Female	7	8 yrs	7	Asian	3			ASA 2	1	Supplemented	5
Patient Status		9 yrs	4	Caucasian	15	Portland Suburbs	14				
New	22	10 yrs	3	Hispanic	9	Other	4				
Recall	8	11 yrs	4								
		12 yrs	3								

Oral Hygiene[4]		Gingivitis[5]		Number of Cavitated Teeth		Restorations		Toothbrushing Frequency		Hours After Brushing Before Visit	
	Number of Patients		Number of Patients		Number of Patients		Number of Patients		Number of Patients		Number of Patients
Good	3	Mild	12	None	9	None	22	3x daily	2	0-1 hrs	8
Fair	13	Moderate	17	1 to 2	7	Amalgam	6	2x daily	21	2-3 hrs	20
Poor	14	Severe	1	3 to 5	7	Composite	1	1x daily	6	>3 hrs	2
				6 or more	7	Sealant	1	<1x daily	1		

[1] A mixture of permanent and primary teeth with different surfaces were selected based on the age of the patient population of 7-12 years, and whether there was a high probability of selected teeth being present at the time of exam. Separate disposable picks (Opalpix; UltraDent Products, Inc.) were used to collect each sample of dental plaque from the buccal surface of the upper first molar (#3 Buccal), the facial surface of the upper left anterior tooth (#9 Facial or #H Facial), the lingual surface of the lower left premolar or second primary molar (#20 Lingual or #K- Lingual), and the lingual surface of the lower central incisors (#25 Lingual). The collection sites were chosen from first preference site to next available preferred site. Collection of plaque from secondary sites occurred in nine out of the 30 participants because of unerupted or early loss of the first tooth site. All participants had at least three of the four site locations available for plaque collection; any individuals with less than three sites available were not included in the study. The collection was conducted by a sweeping action across the entire chosen tooth surface and was not placed subgingival. Each sample pick was then cut and the head placed into sterile transfer tubes that had an anonymous coding, and sealed for transport to the laboratory. The code identified both the sample collection site and the randomly assigned participant number. All examinations and specimen collection were performed at the beginning of every appointment to eliminate potential treatment modalities, such as cleaning or fluoride treatment, that might affect oral environment. A standardized method was used in the collection technique, and one author (SF) was the sole person responsible for the plaque and saliva sample collection, thereby eliminating inter-examiner variability. The OHSU Pediatric Dentistry Clinic generally serves patients of low socioeconomic status. Chart recordings listed medications, including fluoride tablets, oral rinses, and antibiotic use within the last 30 days, last tooth brushing, oral hygiene habits, patient status (new or recall patient), plaque index, and hygiene/tissue condition, or presence of gingivitis and periodontal disease.

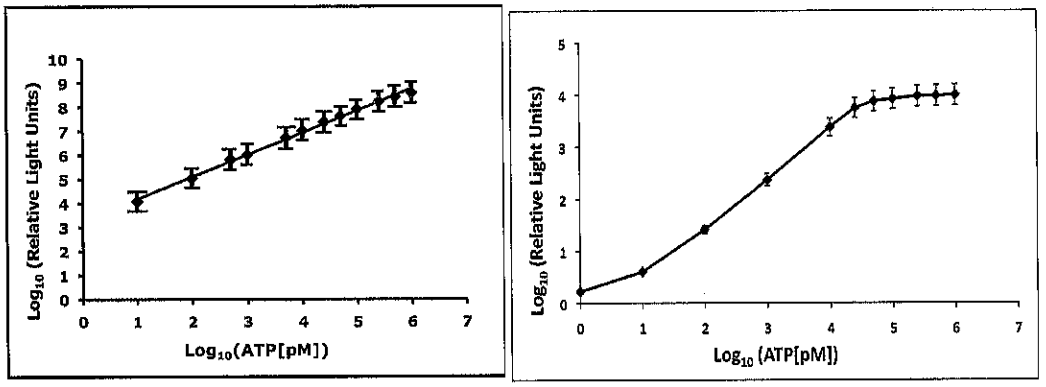
[2] American Association of Anesthesiologists (ASA) physical status classification: ASA 1 – healthy patient with no medical problems; ASA 2 – patient with mild systemic disease; in this case, one patient had mild controlled asthma.

[3] Additional fluoride use means supplemented fluoride in addition to fluoridated toothpaste. Thus, seven patients had access to fluoridated water and five patients had supplemented fluoride from sources other than fluoridated toothpaste. All 30 patients had access to fluoridated toothpaste.

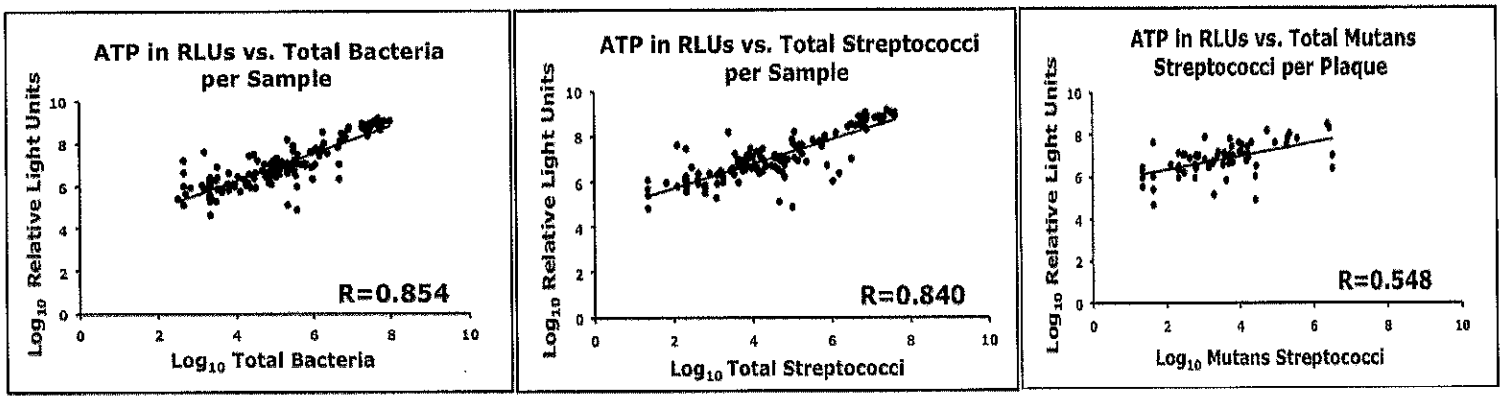
[4] Definitions for oral hygiene: Good – pink gingiva, surfaces free of debris; Fair – red/pink gingiva, surfaces had some debris; Poor – red gingiva, surfaces had definite debris.

[5] Definitions for gingivitis: Mild – marginal gingivitis; Moderate – papillary gingivitis; Severe – spontaneous bleeding of gingiva and/or periodontal disease is evident.

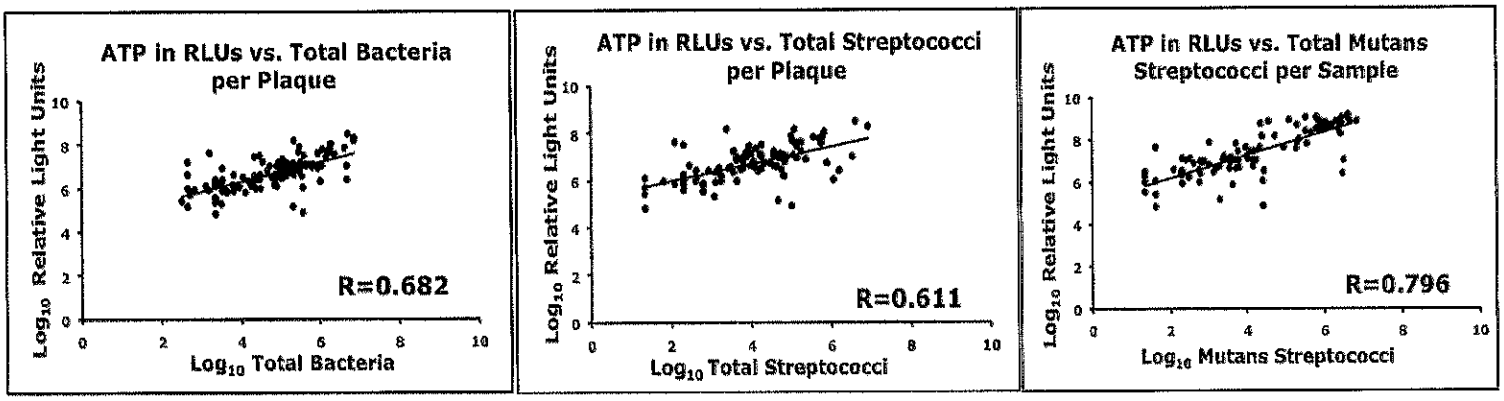
A. Standard Curves Comparing ATP Concentration to Bioluminescence



B. Correlations of ATP Bioluminescence with Bacterial Number (Plaque + Saliva Specimens)



C. Correlations of ATP Bioluminescence with Bacterial Number (Plaque Specimens Only)



D. Correlations of Streptococci and Mutans Streptococci Numbers with Total Bacteria

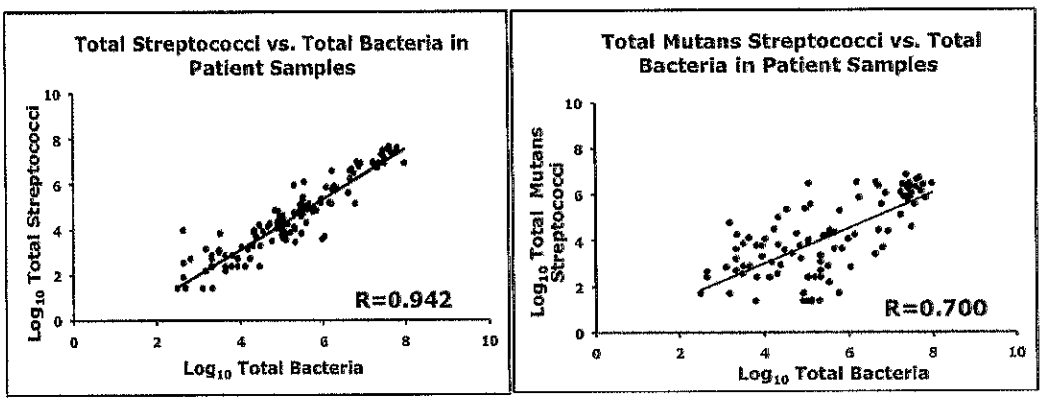
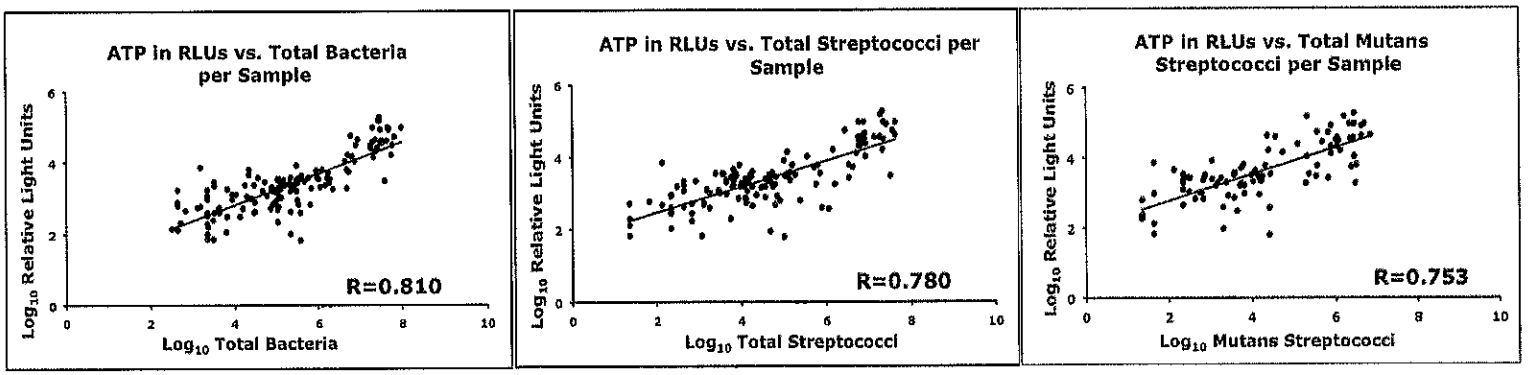


Figure 1 (See Figure Legend section for complete legend to Figure 1).

A. Correlations of ATP Bioluminescence (CariScreen) with Bacterial Number (Plaque and Saliva)



B. Correlations of ATP Bioluminescence (CariScreen) with Bacterial Number (Plaque Only)

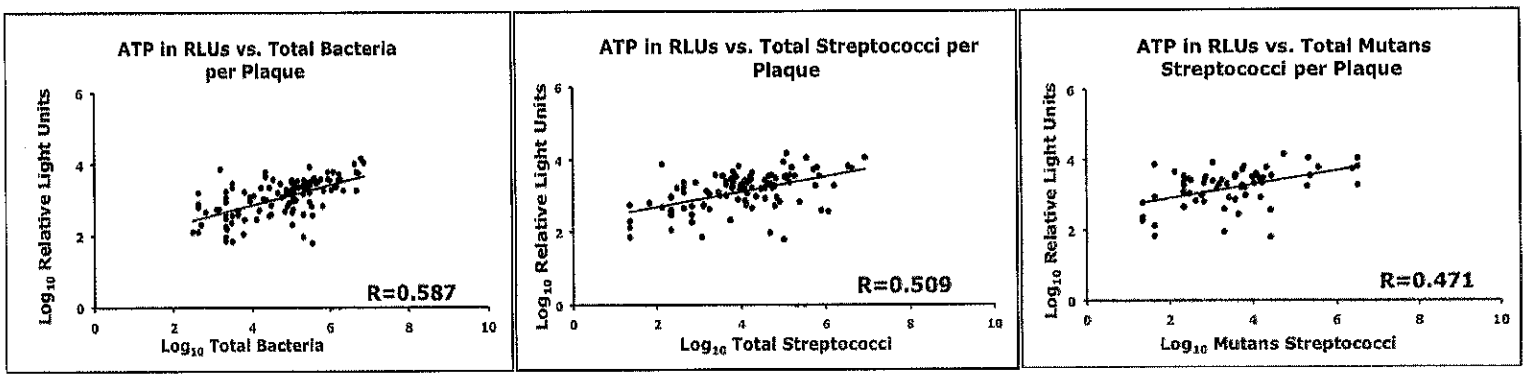


Figure 2

(See Figure Legend section for complete legend to Figure 2).

A.

Lactobacilli CRT Score[1]		Mutans CRT Score[1]		Plaque – Free Surfaces[2]		Active Decay Surfaces[3]	
Score	Number of Patients	Score	Number of Patients	Score	Number of Patients	Score	Number of Patients
1	5	1	4	>80%	0	None	9
2	7	2	3	50-79%	15	1–4	7
3	10	3	10	21-49%	11	5-10	7
4	5	4	10	>20%	4	>11	7

[1]CRT scores for both mutans and lactobacilli numbers were developed and recorded for each saliva specimen. While CRT scores are based on a scale of 1-4 (low to high: score 1: < 10^4 colonies/ml; score 2: 10^4 - 10^5 colonies/ml; score 3: 10^5 – 10^6 colonies/ml; score 4: > 10^6 colonies/ml), and are considered to be semi-quantitative evaluators of mutans streptococci content, the CRT scores obtained for the saliva specimens were consistent with bacterial cell numbers enumerated by direct plating on selective MS agar (unpublished observations). CRT scores were enumerated from only 27 patients because of temporary unavailability of CRT kits from manufacturer and domestic suppliers.

[2] Plaque index scores on selected teeth were determined for each patient. The use of visual examination (without the use of disclosing solution) and tactile feel by an explorer instrument was utilized to detect plaque presence. The number of plaque-free surfaces (out of four possible plaque-free surfaces) were counted for each tooth, and the percentage of plaque-free surfaces were calculated for each patient. After plaque collection and scoring of plaque index, the participant was instructed to chew a paraffin wax tablet and expel saliva into a sterile collection container.

[3] Active decay surfaces were determined by visual examination alone, and not based on use of radiographs.

B.

OHSU Caries Risk Index

1. Lactobacilli CRT Numbers Score

Cell Number	$<10^4$	$\geq 10^4 < 10^5$	$\geq 10^5 < 10^6$	$>10^6$
Score	1	2	3	4

2. S. mutans CRT Numbers Score

Cell Number	$<10^4$	$\geq 10^4 < 10^5$	$\geq 10^5 < 10^6$	$>10^6$
Score	1	2	3	4

3. Plaque Index Score

% Plaque-free Surfaces	≥80%	50-79%	21-49%	≤20%
Score	1	2	3	4

4. Active Surface Decay Score

# Decayed Surfaces	None	1-4	5-10	≥11
Score	1	2	3	4

Final Caries Risk Level

Sum of Scores	4-6	7-9	10-16
Risk Level	Low	Medium	High

C.

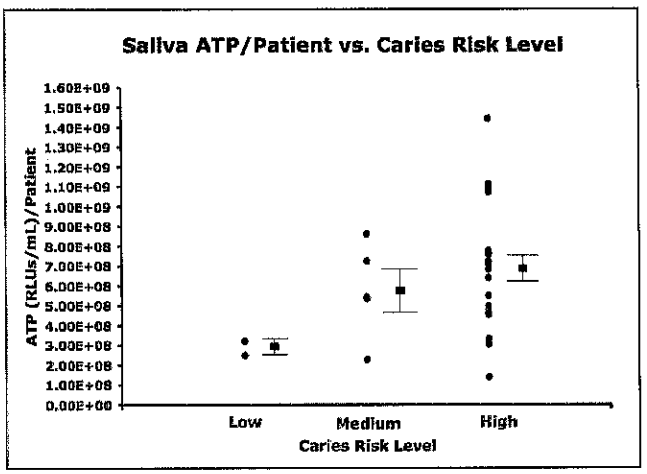


Figure 3

(See Figure Legend section for complete legend for Figure 3).

FIGURE LEGENDS

Figure 1: Using the luciferin substrate and luciferase enzyme, bacterial ATP can be quantitated by measuring the release of visible light (Ronner *et al.*, 1999).

$$ATP + Luciferin + O_2 + Luciferase + Mg^{2+} \text{ -------> } AMP + oxyluciferin + PPi + CO_2 + light\ (560\ nm)$$

The BacTiter Glo Microbial Assay procedure involves adding a single reagent (BacTiter Glo) directly to bacterial cells in medium and measuring bioluminescence. BacTiter Glo contains a proprietary thermostable luciferase and proprietary formulation for extracting ATP from bacteria, and generates a "glow-type" luminescence signal from the luciferase reaction with a half-life of > 30 minutes. For statistical evaluations of the data, we utilized regression analysis and comparison of means. Scatter plot analyses for plaque or saliva specimens linking ATP-driven bioluminescence to bacterial number were developed, and Pearson correlation coefficients (*r* values) were calculated. **A.** ATP standard curves comparing ATP concentration to bioluminescence readouts (RLUs) using the Veritas luminometer (left panel; error bars represent 1 standard error; n = 5 replicate determinations) and CariScreen ATP Meter (right panel; error bars represent 1 standard error; n = 5 replicate determinations). **B and C.** Scatter plot analysis correlating ATP bioluminescence (derived from the Veritas luminometer) versus bacterial cell number for total oral bacteria (left panels), total oral streptococci (middle panels), and mutans streptococci (right panels). **B** depicts data for composite collection of plaque and saliva specimens and **C** depicts data for plaque specimens only. Typical n values equals 30 patients X 4 plaque specimens = 120 plaque specimens plus 30 additional saliva specimens. **D** depicts scatter plot analysis correlating total oral bacterial numbers to total oral streptococci (left panel) and mutans streptococci (right panel). **For B, C, and D:** Data points containing measurements of bacterial number in all plots are the

mean values of 4 replicates (n=4) using plating dilutions exhibiting 50-500 colonies. ATP measurements are tabulated as the mean of 4-5 replicate determinations conducted with the Veritas luminometer. Pearson correlation coefficients (*r* values) are noted in each panel for all correlations. Contrary to other published reports linking plaque weight to ATP content, there were minimal or weak statistical associations between either plaque weight or plaque protein to ATP bioluminescence with *r* values determined at 0 or 0.045, respectively (unpublished observations).

Figure 2: The CariScreen ATP bioluminescence swab collection system and hand-held luminometer (Oral Biotech, Albany, OR) was also utilized in this study; the collection device consisted of a swab and swab holder for collection of oral specimens, and an upper reservoir containing proprietary luciferin, luciferase, and extraction components which can be drained over the swab following collection of specimens. The luciferase contained in the CariScreen system is based on the "flash-type" luminescence signal with RLU readouts peaking at 2 minutes and diminishing over time. For statistical evaluations of the data, we utilized regression analysis and comparison of means. Scatter plot analyses for plaque or saliva specimens linking ATP-driven bioluminescence to bacterial number were developed, and Pearson correlation coefficients (*r* values) were calculated. **A and B.** Scatter plot analysis correlating ATP bioluminescence (derived from the Cariscreen ATP Meter) versus bacterial cell number for total oral bacteria (left panels), total oral streptococci (middle panels), and mutans streptococci (right panels). **A** depicts data for composite collection of plaque and saliva specimens and **B** depicts data for plaque specimens only. Typical n values equals 30 patients X 4 plaque specimens = 120 plaque specimens plus 30 saliva specimens. Data points containing measurements of bacterial number in all plots are the mean values of 4 replicates (n=4) using plating dilutions exhibiting 50-500 colonies. ATP measurements are tabulated as the mean of

4-5 replicate and consecutive determinations conducted in the CariScreen ATP Meter. Pearson correlation coefficients (r values) are noted in each panel for all correlations.

Figure 3: A. Clinical observations, including CRT scores and plaque-free and active decay surfaces, used for determination of OHSU Pediatric Dentistry Caries Risk Index. **B.** Scoring chart for OHSU Pediatric Dentistry Caries Risk Index. **C.** Saliva ATP/patient values versus caries risk level. Caries risk level is a composite score (low risk: range 4-6; moderate risk: 7-9 and high risk: 10-16) of traditional plaque index used by dental professionals, number of active decay (cavitated) surfaces, and CRT results. Individual plaque scores of 1-4 are based on the percentage of plaque-free surfaces. The active decay scores of 1-4 are based on the number of cavitated surfaces. The CRT result, divided as scores for mutans streptococci and lactobacilli, is a graded ranking of bacterial numbers of mutans streptococci and lactobacilli. Values from all categories are then added to develop the composite OHSU Pediatric Dentistry caries risk index score, and then designated as low, moderate, or high caries risk. Trends indicate potential differences and we intend to significantly increase the sample size, including more low risk individuals, in future studies.