

# Scientific Article



## Application of Adenosine Triphosphate-Driven Bioluminescence for Quantification of Plaque Bacteria and Assessment of Oral Hygiene in Children

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**Abstract: Purpose:** Dentistry has undergone a shift in caries management toward prevention and improved oral hygiene and diagnosis. Caries prevention now represents one of the most important aspects of modern dental practice. The purpose of this cross-sectional study was to demonstrate the use of adenosine triphosphate- (ATP-) driven bioluminescence as an innovative tool for the rapid chairside enumeration of oral bacteria (including plaque streptococci) and assessment of oral hygiene and caries risk. **Methods:** Thirty-three pediatric patients (7- to 12-year-old males and females) were examined, and plaque specimens, in addition to stimulated saliva, were collected from representative teeth within each quadrant. Oral specimens ( $n=150$  specimens) were assessed by plating on enriched and selective agars, to enumerate total bacteria and streptococci, and subjected to adenosine triphosphate- (ATP-) driven bioluminescence determinations using a luciferase-based assay system. **Results:** 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. **Conclusions:** 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 oral streptococci, and may be used as a potential assessment tool for oral hygiene and caries risk in children. (*Pediatr Dent* 2010;32:195-204) Received November 17, 2008 | Last Revision April 21, 2009 | Revision Accepted April 22, 2009

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Dental caries is a microbial disease where the principal cariogenic microorganisms have been identified as mutans streptococci (MS) and lactobacilli.<sup>1-4</sup> 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.<sup>5,6</sup> 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 via 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. 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.<sup>1</sup>

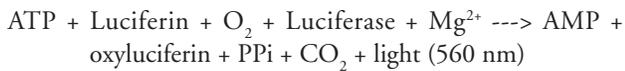
There is a critical need in dentistry to develop better quantitative assessment methods for oral hygiene and to determine patient risk for dental caries, since both disease as well as treatment result in the irreversible loss of tooth structure. Reliable and quantitative determinants of oral hygiene and assessment of caries risk can 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 multifactorial, and caries-risk predictors may be found among the oral microflora, diet, and host, all three of which are essential for caries development.<sup>7</sup> 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.<sup>8,9</sup>

Rapid adenosine triphosphate- (ATP-) driven bioluminescence assays have long been used in the quantitative determination of bacterial numbers—most recently in dental plaque.<sup>10-12</sup> Using the luciferin substrate and luciferase enzyme, bacterial

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ATP can be quantified by measuring the release of visible light<sup>10,11,13</sup> using the following formula:



Bioluminescence assays measuring ATP, flavin mononucleotides, phosphocreatine, and adenylated energy charge have been found to retain high correlations with plaque mass obtained from both humans and animal subjects<sup>10-15</sup> and, by extension have been reflective of bacterial cell number found in dental plaque. Adenylated energy charge (AEC) is a composite measure of the energy potential of oral bacteria, and is a quantifiable measure reflective of actual cell number that is independent of the bacterial growth phase.<sup>15</sup> AEC is an energy composite of ATP, ADP, and AMP, that can be assessed using the enzymes myokinase and pyruvate kinase, and the metabolic substrate phosphoenolpyruvate to drive the conversion of ADP and AMP into ATP.<sup>15</sup> In addition to the measurement of these energy components, new chairside tests for quantification of oral bacteria have been proposed. These include the measurement of lactic acid production on the tongue,<sup>16</sup> which is reflective of bacterial cell number, or by quantitative assessment of cariogenic streptococci using a competitive polymerase chain reaction.<sup>17,18</sup>

The purpose of this study was to demonstrate the use of adenosine triphosphate- (ATP-) driven bioluminescence as an

innovative tool for the rapid chairside enumeration of total oral bacteria, including plaque streptococci. Using plaque and saliva specimens from 33 participants in a cross-sectional study, we compared ATP-driven bioluminescence derived from oral specimens to bacterial number quantified using standard microbiological plating methods. This study tested the hypothesis that ATP measurements obtained from oral clinical specimens can be used as a direct determination of bacterial numbers and, by extrapolation, can serve as a general assessment tool for oral hygiene and potentially for dental caries risk. The vast majority of pediatric patients seen at the Pediatric Dentistry Clinic of Oregon Health & Science University, Portland, Ore, are characterized as high caries risk based solely on socioeconomic status and previous caries history. Consequently, we developed a quantitative caries risk assessment tool for pediatric patients that combined clinical and microbiological evaluations to help better discriminate current risk for dental caries.

**Methods**

**Clinical study patients and clinical observations.** Patients registered for dental care in the Pediatric Dentistry Clinic of Oregon Health & Science University (OHSU) were randomly selected for study inclusion. Thirty-three participants were included in the sample population. The main criterion for selection in this study was age: Selected participants were in the 7- to 12-year-old age group and demonstrated good health

Table 1. PATIENT DEMOGRAPHICS AND CLINICAL OBSERVATIONS\*

Gender	(N)	Age (yrs)	(N)	Ethnicity	(N)	Residence	(N)	Medical history**	(N)	Additional fluoride use†	(N)
Male	23	7	9	African American	3	City of Portland	12	ASA 1	29	In water	7
Female	7	8	7	Asian	3	Portland suburbs	14	ASA 2	1	Supplemented	5
		9	4	Caucasian	15	Other	4				
		10	3	Hispanic	9						
		11	4								
		12	3								
<b>Patient status</b>											
New	22										
Recall	8										
Oral hygiene ‡	(N)	Gingivitis§	(N)	No. of cavitated teeth	(N)	Restorations	(N)	Daily tooth-brushing frequency	(N)	Hours after brushing before visit	(N)
Good	3	Mild	12	0	9	None	22	3x	2	0-1	8
Fair	13	Moderate	17	1-2	7	Amalgam	6	2x	21	2-3	20
Poor	14	Severe	1	3-5	7	Composite	1	1x	6	>3	2
				≥6	7	Sealant	1	<1x	1		

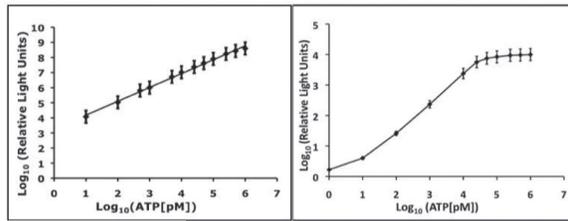
\* The Oregon Health & Science University Pediatric Dentistry Clinic generally serves patients of low socioeconomic status. Chart recordings listed medications, including fluoride tablets, oral rinses, and antibiotic use within the last 30 days, last tooth-brushing, oral hygiene habits, patient status (new or recall patient), plaque index, and hygiene/tissue condition, or presence of gingivitis and periodontal disease.

\*\* 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, 1 patient had mild controlled asthma.

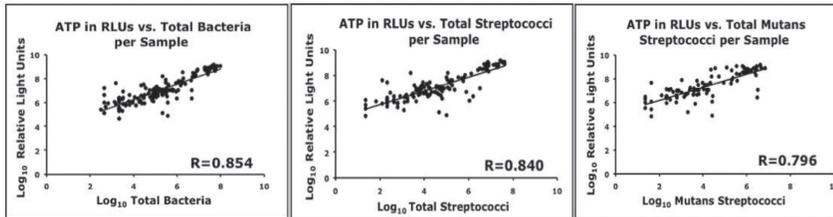
† Additional fluoride use means supplemental fluoride in addition to fluoridated toothpaste. Thus, 7 patients had access to fluoridated water and 5 patients had supplemented fluoride from sources other than fluoridated toothpaste. All 30 patients had access to fluoridated toothpaste.

‡ 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.

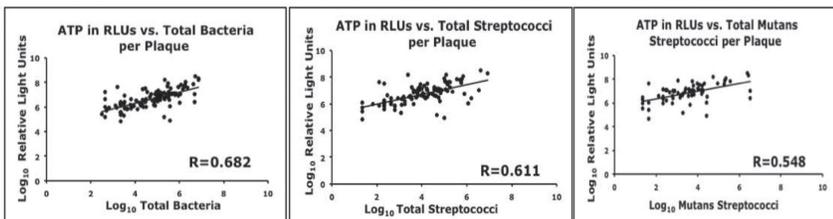
§ Definitions for gingivitis: mild=marginal gingivitis; moderate=papillary gingivitis; severe=spontaneous bleeding of gingiva and/or periodontal disease is evident.



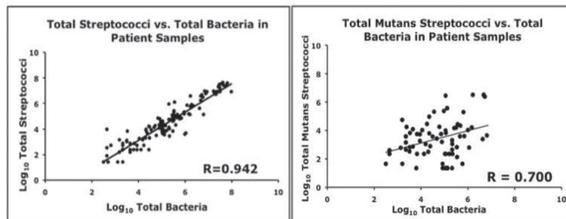
1a. Standard curves comparing ATP concentration to bioluminescence.



1b. Correlations of ATP-driven bioluminescence with bacterial number (plaque + saliva specimens).



1c. Correlations of ATP-driven bioluminescence with bacterial number (plaque specimens only).



1d. Correlations of streptococci and mutans streptococci numbers with total bacteria

Figure 1. (a) Adenosine triphosphate (ATP) standard curves comparing ATP concentration to bioluminescence readouts (RLUs) using the Veritas microplate luminometer (left panel; error bars represent 1 standard error; n=5 replicate determinations) and CariScreen ATP meter (right panel). (b-c) Scatter plot analysis correlating ATP-driven bioluminescence (derived from the Veritas microplate luminometer) vs bacterial cell number for total oral bacteria (left panels), total oral streptococci (middle panels), and mutans streptococci (right panels). Panel B depicts data for collection of plaque and saliva specimens, and panel C depicts data for plaque specimens only. Both panels B and C contain data from specimens collected from 30 participants examined in 2007. n values = 30 patients x 4 plaque specimens = 120 plaque specimens + 30 additional saliva specimens. (d) Panel D depicts scatter plot analysis correlating total oral bacterial numbers to total oral streptococci (left panel) and mutans streptococci (right panel). Data points containing measurements of bacterial number in all plots are the mean values of 4 replicates (N=4) using plating dilutions exhibiting 50 to 500 colonies. ATP measurements are tabulated as the mean of 4 to 5 replicate and parallel determinations conducted in the Turner Biosystems Veritas luminometer. Pearson correlation coefficients (r values) are noted in each panel for all correlations.

were not made available to clinician and laboratory personnel. Patient demographics are displayed in Table 1.

A consent form for routine dental care, currently in use in the OHSU Pediatric Dentistry Clinic, was obtained, and a second consent form specifically for the research study was presented to each participant and parent/guardian. All human subject protocols, consent forms, and specimen collections were reviewed and approved by the Institutional Review Board of OHSU. Chart recordings listed: medications, including fluoride tablets, oral rinses, and antibiotic use within the last 30 days; last known tooth-brushing; oral hygiene habits; patient status (new patient or recall patient); plaque index; and hygiene/tissue condition or presence of gingivitis and periodontal disease. The oral examination included indication of missing teeth, decayed teeth, and restored teeth (Table 1), as well as other information such as partially erupted teeth and restorative materials.

**Plaque index score and caries activity.**

A plaque index score was first completed for each participant on selected teeth without the use of disclosing solution. To simplify our procedure to the basic elements and to reduce additional operator variability in counting plaque surfaces, no disclosing solution was used in the determination of plaque index. Visual examination and tactile probing with an explorer instrument were utilized to assess the presence of plaque. Four surfaces for each selected tooth were examined, and the number of surfaces within each tooth that were determined to be plaque-free were assessed for each participant. Caries activity of the entire dentition was also recorded based on clinical evaluation.

**Plaque specimens collected from specific teeth.** Four teeth representing all 4 quadrants of the mouth were tested in each patient. A mixture of permanent and primary teeth with different surfaces were selected based on the 7- to 12-year-old patient population and whether there was a high probability of selected teeth being present at the time of exam. Teeth such as the maxillary right first molar and mandibular left

and the ability to expel saliva. This age group normally exhibits a wide range of caries experience and plaque levels. 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 (some being high in sucrose). Participants and their parents/guardians were assigned study identifier numbers that

molar were chosen because of their difficulty to brush for most patients, and also because of the close proximity to a salivary duct for one tooth and the distance away from the duct of the other tooth. A maxillary incisor was chosen because of its susceptibility to show enamel demineralization and significant plaque accumulation in children.<sup>19-21</sup> Finally,

a mandibular incisor was chosen because of its close proximity to a salivary gland and the tongue's enhanced cleansing properties.

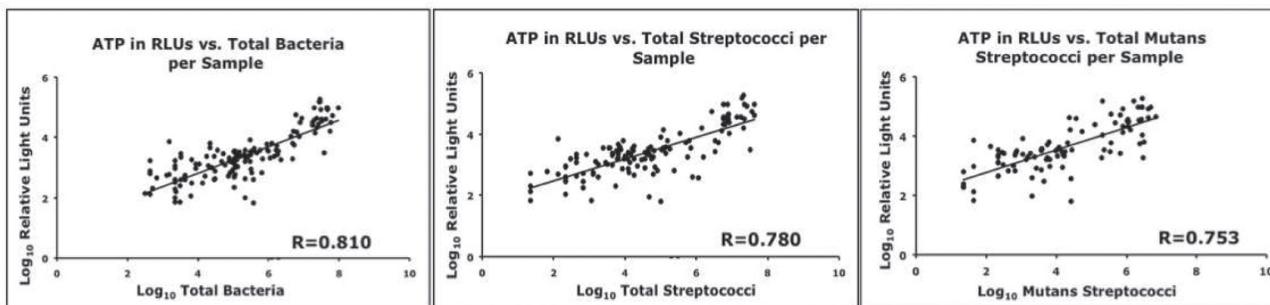
Separate disposable picks (Opalpix, UltraDent Products, Inc, South Jordan, Utah) were used to collect each sample of dental plaque from the: buccal surface of the maxillary first molar (no. 3 buccal); the facial surface of the maxillary left anterior tooth (no. 9 facial or no. H facial); the lingual surface of the mandibular left premolar or second primary molar (no. 20 lingual or no. K lingual); and the lingual surface of the mandibular central incisors (no. 25 lingual). The collection sites were chosen from the first preference site to the next available preferred site. Collection of plaque from secondary sites occurred in nine 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 each head was placed into sterile transfer tubes that had an anonymous coding, and all sealed for transport to the laboratory. The code identified both the sample collection site and the randomly assigned participant number. All examinations and specimen collec-

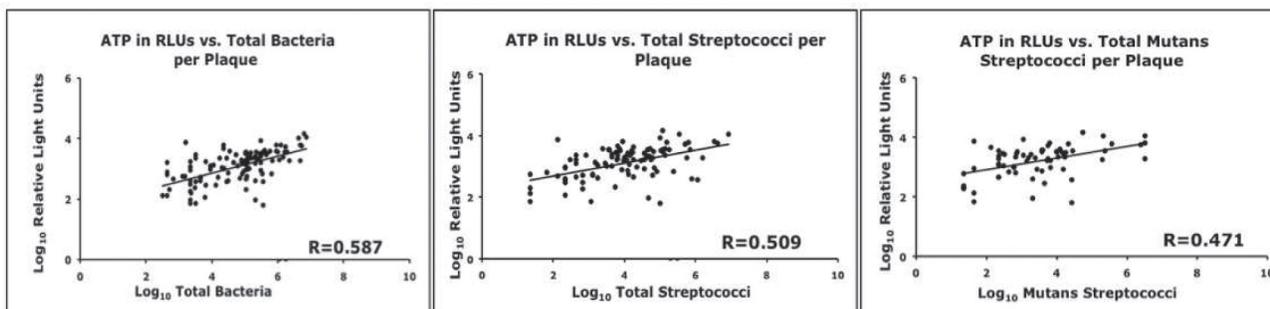
tions were performed at the beginning of every appointment to eliminate potential treatment modalities, such as cleaning or fluoride treatment, that might affect the oral environment. A standardized method was used in the collection technique. One author was responsible for the plaque and saliva sample collection for 30 patients, while another author collected specimens from 3 of the 5 participants identified as low caries risk.

**Collection of stimulated saliva.** After scoring the plaque index and plaque collection, the participant was then given a paraffin wax tablet for chewing. As the saliva was stimulated, the participant expelled saliva into a sterile collection container until a 5-ml sample was collected. The sample was labeled with the unique participant identifier and anonymously coded prior to transfer to the laboratory. Handling of all collected specimens was standardized and conducted by trained lab technicians.

**Culture methods and microbiological identification of plaque and saliva bacteria.** Each plaque specimen was weighed (using Satorius balance calibrated to measure differences as small as 0.1 mg), suspended in 1 ml of phosphate-buffered saline (PBS) with the addition of glass beads, and dispersed by vigorous agitation on a rocker platform (37°C for 5 minutes). Dispersed plaque samples, as well as saliva specimens, were subjected to 10-fold serial dilutions in PBS and then plated on enriched blood agar (product no. 241784-1,



2a. Correlations of ATP-driven bioluminescence with bacterial number (plaque + saliva specimens).



2b. Correlations of ATP-driven bioluminescence with bacterial number (plaque specimens only).

Figure 2. (a-b) Scatter plot analysis correlating ATP-driven bioluminescence (derived from the Cariscreen ATP meter) vs bacterial cell number for total oral bacteria (left panels), total oral streptococci (middle panels), and mutans streptococci (right panels). Panel A depicts data for collection of plaque and saliva specimens, and panel B depicts data for plaque specimens only. This figure displays data from specimens collected from 30 participants examined in 2007. Typical n values = 30 patients x 4 plaque specimens = 120 plaque specimens + 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 to 500 colonies. ATP measurements are tabulated as the mean of 4 to 5 replicate and consecutive determinations conducted in the Cariscreen ATP meter. Pearson correlation coefficients (r values) are noted in each panel for all correlations.

PML Microbiologicals, Wilsonville, Ore) to determine total bacterial numbers. Total oral streptococci were determined by limiting dilution plating on mitis salivarius agar (product no. 229810, Difco, Becton, Dickinson and Company, Sparks, Md), which utilizes high saccharose and vital dyes (ie, crystal violet and bromphenol blue) as selective agents. Mitis salivarius agar was supplemented with potassium tellurite (product no. 211917, Difco, Becton, Dickinson and Company); 1 ml of 1% aqueous potassium tellurite was added to 1 L of mitis salivarius agar prior to pouring of plated medium.

To select and enumerate the subgroup of MS, mitis salivarius agar including potassium tellurite, was also supplemented with bacitracin (10 units/ml, supplied in lyophilized form from Sigma Chemical, St. Louis, Mo). All platings were conducted in quadruplicate, and plates exhibiting colony numbers between 50 and 500 were counted and averaged to determine mean values. As an additional validation in the determination of cariogenic bacteria, we used the commercial CRT Bacteria Test (Vivadent, Lichtenstein, Germany), which incorporates mitis salivarius agar and Rogosa agar in a dual slide test. Caries risk test (CRT) readouts to determine MS or lactobacilli numbers in saliva were interpreted as either less than or greater than  $10^5$  bacteria/ml per manufacturer's instructions.

**ATP-driven bioluminescence and protein concentration determinations of specimens.** ATP contained in bacteria from plaque or saliva specimens was determined with the use of the BacTiter Glo Microbial Cell Viability Assay kit (no. G8231, Promega, Madison, Wis), with ATP-driven bioluminescence measured by the Veritas microplate luminometer (Turner Biosystems, Sunnyvale, Calif). This procedure involves adding a single reagent (BacTiter Glo) directly to bacterial cells in the medium and measuring luminescence. 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 less than 30 minutes. Relative light units (RLUs) were calibrated using a standard curve of ATP (picomolar [pM] concentrations or greater; powdered chemical obtained from Sigma Chemical) and correlated against optical density (or absorbance at a 600 nanometer [nm] wavelength measured with a Novaspec II visible spectrophotometer). The Veritas luminometer has a  $10^5$ -fold dynamic range in RLU readouts.

In parallel luminescence assays, we utilized the CariScreen ATP bioluminescence swab collection system and hand-held luminometer (Oral Biotech, Albany, Ore). 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.

We also determined total protein content in plaque and saliva specimens with the use of Y-PER yeast protein extraction reagent (product no. 78990, Pierce Chemical, Rockford, Ill), the BCA Protein Assay (product A [no. 23221] from Pierce Chemical was combined with product B [no. 1859078] and

200  $\mu$ l of mixture was used per sample), and a 96-well micro titer plate reader. The Y-PER extraction reagent was recommended by the manufacturer to be optimal for extraction of gram-positive bacteria. Protein concentration was calibrated by comparing control solutions containing bovine serum albumin (product no. 23209, Pierce Chemical). Protein assays were analyzed using a Labtec plate reader and Anthos HT3 software 1.06 (Uppsala, Sweden).

### Statistical analyses

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.

### Results

**Experimental overview for the clinical study.** Thirty-three 7- to 12-year-old patients were examined at the OHSU Pediatric Dental Clinic and assessed for oral health. Their dental plaque was collected from representative teeth within each quadrant, followed by collection of a stimulated saliva specimen. Bacterial numbers in dental plaque and saliva were assessed with the use of enriched (blood agar) and selective (mitis salivarius agar without or with bacitracin) agars to enumerate total bacteria, oral streptococci, and MS, respectively. The plaque specimens were also subjected to ATP-driven bioluminescence determinations using both the Turner Biosystems Veritas microtiter luminometer and handheld CariScreen ATP bioluminescence systems. Protein content and weight determinations of plaque and/or saliva specimens were also conducted to determine if these additional factors could be correlated directly to ATP-driven bioluminescence values.

**Calibration of ATP standards to bioluminescence measurements.** Using ATP standards in pM or nM concentrations, bioluminescence (RLUs) standard curves were developed for both the Veritas luminometer and CariScreen ATP meter (Figure 1a, left and right panels, respectively). It was empirically determined that the Veritas luminometer has greater than  $10^5$  dynamic range measuring ATP from 10 pM to 1  $\mu$ M (with RLU readouts from  $10^4$  to  $10^8$  RLUs) and the CariScreen meter has approximately 100-fold dynamic range measuring ATP from 100 pM to 10 nM (with RLU readouts from 15 to 4,000 RLUs). Thus, plaque and saliva specimens were subjected to 10-fold serial dilutions to ensure that at least one of the bioluminescence readouts were measured in the linear portion of the dynamic range for each luminometer. This process was conducted to ensure that the most accurate RLU value, measuring the ATP concentration most reflective of the true cell number, could be compared to bacterial cell numbers enumerated directly by plating on enriched or selective agars.

**Clinical observations from patient population.** Information from patients was collected concerning gender, age, ethnicity, residence, and medical history (Table 1). Fluoride use, oral hygiene, presence of gingivitis, numbers of teeth with cavities, numbers of active caries surfaces, and numbers of plaque-free surfaces were also noted.

**Strong statistical correlation linking ATP-driven bioluminescence to total oral bacteria and total oral streptococci.** Saliva and plaque specimens were collected from the selected teeth of 33 patients (1 plaque specimen from each mouth quadrant; N=4 plaque specimens per patient), and ATP-driven bioluminescence was measured from each specimen. Using serial dilution plating of each oral specimen, quantification was conducted for total bacteria on enriched medium (blood agar) and total streptococci and MS on selective medium (mitis salivarius agar), in the absence or presence of bacitracin, respectively. When ATP-driven bioluminescence values, using the Turner Bio-systems Veritas luminometer, were determined using plaque and saliva specimens, strongly significant Pearson correlation coefficients of 0.854, 0.840, and 0.796 were determined for total oral bacteria, total oral streptococci, and MS,

respectively (with 1.0 being a perfect correlation; see Figure 1b; left, middle and right panels, respectively).

When these ATP-driven bioluminescence readings were analyzed using plaque specimens only, which in this case reduces the statistical power because of a smaller sample set, significant Pearson correlation coefficients of 0.682, 0.611, and 0.548 were still identified for total oral bacteria, total oral streptococci, and MS, respectively (Figure 1c; left, middle and right panels, respectively).

When scatter plot analyses were conducted correlating total oral bacteria with either total oral streptococci or MS (Figure 1d; left and right panel, respectively), increasing numbers of total oral bacteria were found to track linearly with total oral streptococci in a strongly significant relationship ( $r=0.94$ ), and also to a lesser but still significant degree with MS ( $r=0.70$ ).

**A.**

Lactobacilli CRT Score <sup>1</sup>		Mutans CRT Score <sup>1</sup>		Plaque – Free Surfaces <sup>2</sup>		Active Decay Surfaces <sup>3</sup>	
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

<sup>1</sup> CRT scores were enumerated from only 27 patients because of temporary unavailability of CRT kits from manufacturer and domestic suppliers.

<sup>2</sup> 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 (1 of 4 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.

<sup>3</sup> 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 <sup>4</sup>	≥10 <sup>4</sup> <10 <sup>5</sup>	≥10 <sup>5</sup> <10 <sup>6</sup>	>10 <sup>6</sup>
Score	1	2	3	4

**2. S. mutans CRT Numbers Score**

Cell Number	<10 <sup>4</sup>	≥10 <sup>4</sup> <10 <sup>5</sup>	≥10 <sup>5</sup> <10 <sup>6</sup>	>10 <sup>6</sup>
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.**

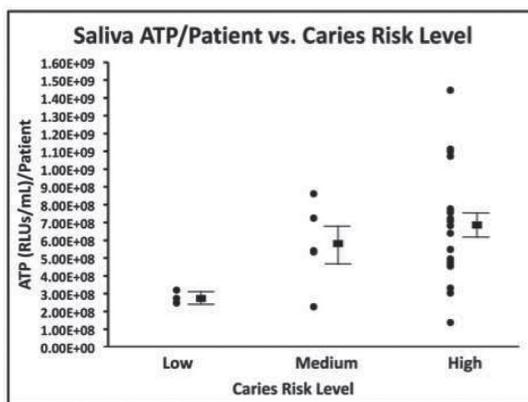


Figure 3. (a) Clinical observations, including caries risk test (CRT) scores and plaque-free and active decay surfaces, used for determination of Oregon Health & Science University's pediatric dentistry caries risk index (OHSU PD CRI). (b) Scoring chart for OHSU PD CRI. (c) Saliva adenosine triphosphate (ATP)/patient values vs caries risk level. Caries risk level is a composite score (low risk range=4-6; moderate risk range=7-9; and high risk range=10-16) of traditional plaque index used by dental professionals, number of active decay (cavitated) surfaces, and CRT results. Individual plaque scores of 1 to 4 are based on the percentage of plaque-free surfaces. The active decay scores of 1 to 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 PD CRI score, and then designated as low, moderate, or high caries risk. Panel C displays data from 33 participants, including 30 participants seen in 2007 and 3 additional low caries risk patients seen in 2008 (n=5 for low caries risk individuals).

Similar ATP-driven bioluminescence relationships were found using the handheld CariScreen ATP meter. 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 MS (Figure 2a-b;  $r=0.81$ ,  $0.78$  and  $0.75$ , respectively, using plaque and saliva specimens, and  $r=0.59$ ,  $0.51$ , and  $0.47$ , respectively, using plaque specimens only).

Thus, ATP-driven bioluminescence is highly predictive of the numbers of total oral bacteria and total oral streptococci. 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.

CRT scores for both MS and lactobacilli numbers were also developed and recorded for each saliva specimen. While CRT scores are based on a scale of 1 to 4 (low to high: scores 1 and 2 represent  $<10^5$  colonies and 3 and 4 represent  $>10^5$  colonies) and are considered to be semiquantitative evaluators of MS content, the CRT scores obtained for the saliva specimens were consistent with bacterial cell numbers enumerated by our direct plating on selective agars (Table 1 and unpublished observations).

**Minimal statistical association of plaque weight and plaque protein to ATP-driven bioluminescence.** Contrary to the published report of Robrish et al.<sup>10</sup> linking plaque mass to ATP content, we have found that there were minimal or weak statistical associations between either plaque weight or plaque protein to ATP-driven bioluminescence with  $r$  values determined at 0 or 0.045, respectively. Robrish et al.<sup>10</sup> estimated levels of extractable ATP from dental plaque obtained from monkeys as a measure of viable cell mass, and estimated total cell mass by measuring protein content. In our study, the lack of statistical association between either plaque weight or plaque protein to ATP-driven bioluminescence was presumably due to the complex and variable nature of plaque biofilm being composed of not only microorganisms, but also nonprotein-based extracellular matrix as well as food detritus.

Interestingly, when protein determinations were conducted for the complete set of plaque and saliva specimens and then compared to corresponding ATP values, the  $r$  value increased to 0.598. There may have been statistical bias in this increased  $r$  value, because the scatter plot analysis of this data showed two segregated distributions for either plaque or saliva specimens. All plaque specimens had relatively low protein content, while all saliva specimens had relatively high protein content (unpublished observations), resulting in the connection of an arbitrary line linking the two concentrated distributions.

## Discussion

**Criteria used for development of OHSU pediatric dentistry caries risk index.** In the patient population at the OHSU Pediatric Dental Clinic, all children would be considered high risk according to the currently recommended AAPD caries assessment tool (CAT). Furthermore, the AAPD CAT as well as most other assessment tools has a previous caries-related his-

tory of bias against potential reassessment of future caries risk. To be able to quantify and better rank our study population, a composite of quantifiable clinical and microbiological evaluation criteria was included to identify current risk for dental caries.

Three index factors were used to develop the OHSU pediatric dentistry caries risk index (CRI). The first factor was visible bacterial plaque without the use of disclosing solution. Alaluusua and Malmivirta<sup>22</sup> demonstrated that visible plaque on the labial surfaces of maxillary incisors was strongly associated with caries development. In that study, the best indicator of caries risk was visible plaque compared to other potential indicators, including the use of a nursing bottle, mother's caries prevalence, and mother's salivary level of MS. Wendt et al.<sup>23</sup> also determined that children with no visible plaque at 2 years old had greater chances of remaining caries-free until 3 years old, compared to children with visible plaque. Although in some studies supragingival plaque accumulation has not been highly correlated with caries experience,<sup>24-27</sup> studies by Lindhe et al.<sup>28</sup> and Poulsen et al.<sup>29</sup> showed that professional plaque removal could prevent caries, thus establishing dental plaque as a significant and probable risk factor for dental caries.<sup>30</sup>

The second index factor was presence of active caries. In a systematic review of the literature, Zero et al.<sup>1</sup> stated that the best predictor for caries in primary teeth was previous caries experience. For permanent teeth in children and adolescents, DMF scores and pit and fissure morphology were the most important indicators. Zero et al.<sup>1</sup> stated that previous caries experience was an important predictor in most models tested for primary, permanent, and root surface caries. Li and Wang<sup>31</sup> demonstrated that caries in the primary dentition is predictive of caries in the permanent dentition. To illustrate the continuum of cariogenic disease, Greenwell et al.<sup>32</sup> 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 MS and lactobacilli. In the current AAPD CAT model, microbiological testing has been highly recommended. The importance of MS in the development of dental caries has been reviewed extensively.<sup>30,33-35</sup> Studies in humans have shown that subjects with active caries tend to harbor higher numbers of MS and lactobacilli in their saliva and plaque than caries-free individuals.<sup>30,33,36-41</sup> In longitudinal studies, it has been demonstrated that there is an increase over time in the numbers of MS and lactobacilli associated with the onset and progression of caries.<sup>42-45</sup>

Another important risk factor in young children is the age of the children at time of colonization with MS. Children at infancy with high levels of MS, compared to older children, have more severe caries in the primary dentition.<sup>22,46,47</sup> Most infants acquire MS between 19 and 33 months old, with some as early as 8 to 10 months old. Consequently, the AAPD, American Dental Association, and the American Association of Public Health Dentistry all recommend initial oral evaluation of a child by 1 year of age. The American Academy of Pediatrics recommends referral to a dentist by age 1 if the mother has a high caries rate, the child has demonstrable caries, plaque, demineralization, and/or staining, the family is of low

socioeconomic status, the child has special care needs, and if the child is a later order offspring.<sup>8</sup>

#### Quantitative formulation of OHSU pediatric dentistry

**CRI.** Thus, the OHSU pediatric dentistry CRI score is composed of measures consisting of: (1) traditional plaque index used by dental professionals; (2) number of cavitated surfaces; and (3) CRT (Vivadent) results enumerating MS and lactobacilli numbers (Figure 3a-b). Values from all 3 categories are then added to develop the composite caries risk score and designated as low, moderate, or high caries risk using the following equation:

$$\text{CRI score} = \text{Plaque score } (\frac{1}{4}) + \text{active surface decay score } (\frac{1}{4}) + \text{CRT (includes lactobacilli and } \textit{Streptococcus mutans} \text{ scores)} (\frac{1}{2})$$

Thus, the ranges of composite scores for low, moderate, and high caries risk are 4 to 6, 7 to 9, and 10 to 16, respectively (Figure 3a-b). By combining the 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-driven bioluminescence identified in this study.

**Relationship between ATP-driven bioluminescence, oral hygiene, and caries risk.** There appears to be stepwise increase in ATP-driven bioluminescence (obtained from saliva specimens) for any given patient when plotted against the broad categories of patients exhibiting low and moderate/high caries risk based on our OHSU pediatric dentistry CRI score (Figure 3c). Even though most individuals seen at the OHSU Pediatric Dental Clinic were considered high caries risk, the sample size for low caries risk individuals was sufficient (N=5) for making comparative determinations. Trends indicated potential differences, and we intend to significantly increase the sample size, including more low-risk individuals, in future studies.

In addition, based on ATP-driven 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. We were also unable to discriminate differences between primary and permanent teeth for individuals examined in our study and plan to explore these potential differences in future studies.

While we understand that our ATP-driven bioluminescence values measure total oral bacterial number and not necessarily cariogenic microorganisms, this assay represents an excellent assessment tool for the efficacy of oral hygiene and potentially for the use of interventional procedures, including the use of antibacterial mouthrinses. While the use of the laboratory luminometer may be cost-prohibitive for most dental practices, the use of the hand-held CariScreen ATP meter is simple-to-use at chairside, relatively inexpensive, and when used appropriately, is predictive of the total numbers of oral bacteria contained in dental specimens. By extrapolation, based on our statistical analysis of a population of patients with high plaque bacterial load, these individuals also contain compar-

tively high streptococci and cariogenic MS numbers. While not absolutely definitive for cariogenic streptococci, the use of ATP-driven bioluminescence may potentially be used for assessment of plaque bacterial number and plaque load as an indirect indicator of dental caries risk.

#### Conclusions

Based on this study's results, the following conclusions can be made:

1. This study has provided validation that adenosine triphosphate-driven bioluminescence may be used as a quantitative assessment tool for the determination of total oral bacteria, including plaque bacteria, and can be used to assess the efficacy of oral hygiene. ATP-driven bioluminescence was measured using commercially available systems: one using a bench-top luminometer and the second using a self-contained swab collection system and hand-held luminometer. The ability of the latter system was shown to provide rapid and reliable chairside measurements.
2. The strong statistical correlation between total oral bacteria and oral streptococci, including cariogenic streptococci, infers that ATP-driven bioluminescence may also potentially serve as an indirect assessment determinant of dental caries risk.
3. Most pediatric patients seen at the OHSU Pediatric Dentistry Clinic are characterized as high caries risk based solely on socioeconomic status and previous caries history. Consequently, we developed a quantitative caries risk assessment tool for pediatric patients that combined clinical and microbiological evaluations to help better discriminate current risk for dental caries.
4. The use of ATP-driven bioluminescence has broad implications in dentistry and medicine and can be used translationally in the clinic to determine the efficacy of interventional therapies, including the use of mouthrinses, and perhaps in the detection of bacterial infections in periodontal and other infectious diseases.

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