Screening of a Pediatric and Adult Clinic Population for Caries Pathogen *Scardovia Wiggsiae*

Lindsay Row*/ Matthew R Repp**/ Karl Kingsley***

Dental caries remains one of the most common morbidities worldwide, with research focusing on the most prevalent cariogenic bacteria including Streptococcus, Actionmyces and Lactobacillus species. However, recent evidence has begun to reveal novel pathogens that may alter current understanding of caries risk, including the recently discovered Gram-positive bacillus Scardovia wiggsiae. ***Study Design****: This prospective, cross-sectional study collected saliva from healthy adults and children for DNA isolation and PCR screening (n=149) in order to evaluate the prevalence of this potential oral pathogen.* ***Results****: This analysis revealed that although half of these samples harbored traditional pathogens (S. mutans), approximately one quarter also harbored detectable levels of Scardovia. Further analysis using semi-quantitative methods, however, suggested only a small percentage had elevated (high) microbial burden (5.4%). These data suggest that cariogenic microbial composition among some patients may differ significantly, with some patients harboring comparatively larger percentages of Scardovia within their oral microbial flora.* ***Conclusions****: A complete understanding of the microbial interactions that facilitate or hinder the growth of Scardovia within the oral cavity remains largely unknown, suggesting the need for long-term studies to more fully elucidate demographic or health parameters, as well as the potential disease risk, associated with this organism.*

Key-words: Scardovia wiggsiae, caries, pediatric, dental, saliva

INTRODUCTION

Dental cavities or caries is among the most prevalent of health conditions in the world today with current estimates now suggesting 2.4 billion new cases, including more than 620 million children with untreated caries.[1] Considerable research has been undertaken to improve the effectiveness of oral health literacy and oral health education among children to reduce this burden, although many barriers remain and much greater investment may be necessary over the long term to effect greater change.[2]These research endeavors have produced vital and necessary health information to improve childhood diet and nutrition, specifically to reduce caries risk and morbidity.[3]

*Lindsay Row, BS, Fourth-year dental student, University of Nevada – Las Vegas, School of Dental Medicine.
**Matthew R Repp, BS, Pre-medical student, Arizona State University.
***Karl Kingsley, PhD, MPH, Associate Professor, University of Nevada – Las Vegas, School of Dental Medicine.

Send all correspondence to:
Karl Kingsley
University of Nevada, Las Vegas - School of Dental Medicine
1001 Shadow Lane, B313
Las Vegas, Nevada 89106
Phone:(702) 774-2623
Fax:(702) 774-2721
E-mail: Karl.Kingsley@unlv.edu

New studies are beginning to reveal novel pathogens that may confer additional risks and might alter the current understanding of caries screening. For example, although numerous studies have demonstrated the causal link between *Streptococcus mutans* and caries lesions, more recent evidence suggest caries pathogens constitute part of a much larger oral microbial community that may disproportionately influence caries risk when minor constituents become imbalanced – a process known as dysbiosis.[4,5] In fact, many of these microbial species are well-known and have been extensively studied in the context of the cariogenic process, including *Streptococcus*, *Lactobacillus*, *Veillonella* and *Actinomyces* species.[6] However, several recent studies have demonstrated the presence of newly characterized cariogenic pathogen, the anaerobic Gram-positive bacillus *Scardovia wiggsiae.*[7-9]

More recently, this group performed a retrospective analysis of previously collected saliva samples from both teenagers and adults to screen for the presence of this organism, which was more prevalent among minority patients.[10] Although these data complement a growing body of evidence using saliva as a non-invasive screening matrix for oral pathogens and cariogenic organisms, the retrospective nature of these studies precludes finding additional oral and systemic health information to provide context and caries history for these patients.[11-13]

The main objective was to prospectively collect saliva samples from the pediatric and adult clinic populations of a public dental school to screen for *Scardovia wiggsiae,* while simultaneously collecting multiple measures of oral and systemic health – including body mass index (BMI), decayed missing and filled teeth (DMFT)

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score, age, ethnicity, sex, caries risk and other medical concerns. The results of the molecular screening will then be compared with these variables to determine any potential associations with the presence of *Scardovia wiggsiae*.

MATERIALS AND METHOD

The protocol for this study "The Prevalence of Oral Microbes in Saliva from the UNLV School of Dental Medicine pediatric and adult clinical population" (Protocol#1305-4466M) was reviewed and approved by the University of Nevada, Las Vegas (UNLV) Office for the Protection of Research Subjects (OPRS) Institutional Review Board (IRB). A cross sectional (observational) prospective research design was used to conduct this study. A convenience sample of pediatric patients was recruited from the University of Nevada Las Vegas (UNLV) School of Dental Medicine (SDM) clinics. Pediatric dental residents recruited parents or guardians of UNLV pediatric dental patients between the ages of 3- 17 years, who provided Informed Consent for their child to participate in this study. Pediatric Assent from each patient was also obtained prior to collection of demographic data and saliva samples. Exclusion criteria for participation were limited to patients who declined to participate, any parent or guardian who declined to participate, or any child that was not a patient of record at UNLV-SDM. In addition, saliva samples were also collected from adults (> 18 years old) from a convenience sample at UNLV-SDM during their dental visit. Informed consent was required and was conducted onsite. Subjects younger than 18 years of age and subjects that declined to participate were excluded. Consented dental patients were given a sterile saliva 50 mL collection container for one sample. Samples were stored on ice until transfer to a biomedical laboratory for screening and analysis. Each of these samples was given a unique, randomly generated number to prevent research bias and any identifying information from being disclosed. The patient demographic and health information was also collected and given the matching randomly generated number for analytical purposes, but no patient-specific identifying information was available to any research team member.

Cell counting and DNA isolation

All saliva samples, which contained both shed epithelial cells and bacterial cells, were centrifuged for 10 minutes at 2,100 g (RCF) and the pellet washed with 1X phosphate-buffered saline (PBS) (HyClone: Logan, Utah, USA) and resuspended in 5 mL of 1X PBS. Epithelial cell number was determined using Trypan Blue (Fisher Scientific: Fair Lawn, New Jersey, USA) using a Zeiss Axiovert 40 inverted microscope (Carl Ziess, Inc: Thornwood, New York, USA) and a hemacytometer (Fisher Scientific: Fair Lawn, New Jersey, USA). To determine if any samples harbored the cariogenic pathogen of interest - SW, DNA was isolated from the saliva sample using the GenomicPrep DNA isolation kit (Amersham Biosciences: Buckinghamshire, United Kingdom) and the procedure recommended by the manufacturer for blood and tissue, which recommends a minimum of 3.5×10^5 cells.[11-13] DNA was resuspended in 50 µL and stored in DNA Hydration Solution (Amersham Biosciences: Buckinghamshire, United Kingdom) at 4C. DNA purity was calculated using ratio measurements of absorbance at 260 and 280 nm (A260/A280 ratio between 1.47 and 2.0).

Polymerase chain reaction (PCR)

DNA from each sample was then used to perform PCR with the Fisher exACTGene complete PCR kit (Fisher Scientific: Fair Lawn, New Jersey, USA) and a Mastercycler gradient thermocycler (Eppendorf: Hamburg, Germany) using the following primers for *S. wiggsiae*, *S. mutans*, *P. gingivalis*, 16S rRNA and glyceraldehyde-3- phosphate dehydrogenase (GAPDH) (SeqWright: Houston, Texas, USA) .[7,8,12]

GAPDH forward primer, ATCTTCCAGGAGCGAGATCC;
GAPDH reverse primer, ACCACTGACACGTTGGCAGT;

16S rRNA universal primer, ACGCGTCGACAGAGTTT-GATCCTGGCT;
16S rRNA universal primer, GGGACTACCAGGGTATCTAAT;

S. mutans forward primer, GCCTACAGC TCAGAGATGC-TATTCT;
S. mutans reverse primer, GCCATACACCACTCATGAATTGA;

P. gingivalis forward primer, TACCCATCGTCGCCTTGGT;
P. gingivalis reverse primer, CGGACTAAAACCGCATACACTTG;

S. wiggsiae forward primer, GTGGACTTTATGAATAAGC;
S. wiggsiae reverse primer, CTACCGTTAAGCAGTAAG;

DNA standard: GAPDH

DNA standards obtained from standardized control cells, human gingival fibroblasts ($0.3\text{-}0.5 \times 10^6$ cells/mL), approximating the range of cell concentrations observed in the saliva samples were used to establish the minimum threshold (CT) and saturation (CS) cycles required for calibration and concentration comparisons using relative endpoint PCR (RE-PCR). GAPDH signal detection above background or CT required a minimum of ten cycles (C10), with saturation or CS observed at C50. Based upon these data, RE-PCR was performed at C30, above the lower detection limit but below the saturation limit.

One mg of template DNA was then used for each reaction. The initial denaturation step ran for three minutes at 94°C. A total of 30 amplification cycles (C30) were run, consisting of 30 second denaturation at 94°C, 60 seconds of annealing at 58°C, and 30 seconds of extension at 72°C. Final extension was run for five minutes at 72°C. The PCR reaction products were separated by gel electrophoresis using Reliant 4% NuSieve® 3:1 Plus Agarose gels (Lonza: Rockland, Maine, USA). Bands were visualized by UV illumination of ethidium-bromide-stained gels and captured using a Kodak Gel Logic 100 Imaging System and 1D Image Analysis Software (Eastman Kodak: Rochester, New York, USA).

Standard curves: semi-quantitative analysis

The human oral gingival fibroblast cell line HGF-1 (CRL-2014) was obtained from American Type Culture Collection (ATCC; Manassas, VA). Cell number was determined from a small aliquot using Trypan Blue (Fisher Scientific), a Zeiss Axiovert 40 inverted microscope (Gottingen, Germany) and a standard hemacytometer (Fisher Scientific). Dilutions to approximate final concentrations of 5.0×10^6, 10^5, 10^4 and 10^3 cell/mL were prepared to establish RE-PCR standards from these DNA extractions.

In addition, the oral bacterial cell lines *Streptococcus mutans*

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(*S. mutans* or SM) (NCTC-10449) and *Porphyromonas gingivalis* (*P. gingivalis* or PG) (FDC-381) were also obtained from ATCC (Manassas, VA). In brief, cells were thawed, streaked and cultured on their respective agar plates from Difco (Sparks, MD) according the manufacturer protocol.[12] Colonies of each were then plated and grown overnight at 37º C on Trypticase soy agar; SM plates were supplemented with 5% defibrinated sheep's blood and PG plates were supplemented with 1% yeast extract. Single plate colonies were then selected and inoculated into liquid broth cultures; Trypticase soy broth for SM and supplemented tryptic soy broth for PG and then incubated overnight at 37º C. Aliquots of bacterial suspensions were subsequently used to inoculate growth standards.

Standard curves were created using spectrophotometric absorbance measurements of optical density (OD) at 650 nm and enumeration of colony forming units (CFU).[10,12] Turbidity resulting in an OD of 0.8 corresponded to 5.0 x 10^7 CFU/mL for both bacterial cell lines used. Serial dilutions were prepared for final concentrations of 5.0 x 10^6, 10^5, 10^4 and 10^3 CFU/mL to establish RE-PCR standards for SM and PG corresponding with the most current understanding of microbial saliva concentrations as biomarkers for disease (including caries) risk, which are: 10^6 CFU/mL = very high risk; 10^5 CFU/mL = high risk; 10^4 CFU/mL = moderate risk, and < 10^3 CFU/mL = normal or average risk.[12,14,15]

Statistical analysis

To determine the appropriate sample size for this type of PCR screening for microbial composition using DNA extracted from saliva, the recovery rate from the sample-limited step of DNA extraction was used (90-95%) to establish the minimum expected difference of 0.10 or 10%.[16] Using a significance level of $\alpha = 0.05$ and a power $p = 0.80$, a minimum sample size of fifty (N = 50) was calculated.[17] Descriptive statistics regarding the sample population were reported, and chi-square (c^2) analysis was performed to determine any significant differences in demographics between the sample group and the clinic population.

RESULTS

Patients that consented to be in the study were grouped based upon their age (pediatric < 18 years of age; adult > 18 years of age) for comparative analysis with the overall respective clinic populations (Table 1). Pediatric patient samples (n=81) contained slightly more females (54.3%) than males (45.7%), which was not significantly different from their distribution among the pediatric clinic population (p=0.14). However, the proportion of pediatric samples from minority patients (80.2%) was slightly, but significantly, lower than that of the pediatric clinic (89.1%) in general (p<0.01). Despite this difference, the proportion of Hispanics, which constitute the greatest percentage of pediatric clinic patients, was approximately the same from pediatric samples and the pediatric clinic population.

The adult patient samples (n=81) had more males (56/8%) than females (43.2%), which was significantly different from their percentages within the main clinic patient population (p<0.001). In addition, the percentage of samples from minority patients (39.5%) was lower than the overall clinic patient population (59.2%), which was statistically significant (p<0.001). The overall composition of adult and pediatric samples revealed a nearly equal proportion of females and males (48.7%, 51.2%, respectively), with a majority of samples derived from minority patients (59.9%).

The saliva samples were then processed to isolate DNA and screen for the presence of both human and bacterial DNA (Table 2). DNA was successfully recovered from 76/81 of the pediatric samples, yielding a recovery rate of 93.8%. The recovery rate from the adult patient samples was slightly lower (90.1% or n=73/81). These values were within the acceptable range for recovery (>90%) according to the manufacturer, as well previous comparable studies. [10-13]. DNA concentrations ranged from an average of 171.29 ng/uL from the pediatric samples to 218.3 ng/uL from the adult patient samples. DNA purity was calculated using ratio measurement of absorbance at 260 and 280 nm (A260/A280 ratio), which ranged between 1.47 and 2.0. The processing of all samples using PCR to screen the pediatric and adult samples revealed the presence of both human and bacterial DNA.

Standards of genomic DNA extracted from bacterial and human standards were used to confirm the detection thresholds (C_T) and saturation (C_S) cycle limits (Figure 1). PCR using SM and PG standards resulted in strong, positive curvilinear correlations between band intensity and DNA standards from SM and PG samples with increasing CFU/mL concentrations, as well as from HGF-1 cells. The observed detection threshold for 5.0 x 10^3 cells/mL or CFU/mL was 30 cycles (C30), with saturation observed at 50 cycles (C50). Correlation of band intensity at C30 with cell number from HGF-1 (human) standards was R^2=0.9879, SM was R^2 = 0.992, and PG was R^2=0.9947, providing a semi-quantitative method for estimating cell number/mL.

All samples were then screened using PCR, which screening revealed the presence of *Scardovia wiggsiae* (SW) in approximately one quarter of all samples tested (Table 3). More specifically, 26.3% of pediatric and 24.7% of adult samples had detectable levels of *Scardovia*. Moreover, nearly half of these samples also harbored *S. mutans* – including 47.4% of pediatric samples and a significantly greater percentage among (56.2%) among the adult samples. Finally, *P. gingivalis* was detectable in a lower percentage of pediatric (15.8%) versus adult (24.7%) samples, which was also statistically significant (p<0.01).

Further analysis of the PCR screening results using signal band intensity (SBI) allowed for sorting of samples into risk categories (Figure 2). The highest percentage of SM-positive samples (n=36) were in the low-risk (LR) category, while the vast majority of samples were below the limit of detection (bLOD) suggesting the percentage of patients with normal risk was nearly three quarters or 72.5% (n=108/149). Many fewer SM-positive samples were found in the moderate risk (MR), high risk (HR) and very high risk (vHR) categories (n=17, 11, and 13, respectively), suggesting 27.5% of patients were at some level of increased disease risk. A similar distribution was found among PG-positive samples, although an even larger percentage within the normal range (85.2% or n=127/149). Finally, although culturing methods have not been standardized for SW, categorization of the results using SBI provided a semi-quantitative method for estimating CFU/mL that revealed the overwhelming majority of samples within the normal (LR or bLOD) risk categories (n=141/149), with an estimated n=8 or 5.4% corresponding to the higher/increased risk categories associated with SM and PG.

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Table 1. Demographic analysis of study samples

	Pediatric samples	Pediatric clinic	Statistics	Adult samples	Main clinic	Statistics	Overall (combined)
Females	(n=44) 54.3%	52%	c2=2.119 d.f.=1	(n=35) 43.2%	49.4%	c2=15.378 d.f.=1	(n=79) 48.7%
Males	(n=37) 45.7%	48%	*p*=0.1454	(n=46) 56.8%	50.6%	*p*<0.001	(n=83) 51.2%
White	(n=16) 19.8%	10.9%	c2=81.560 d.f.=1	(n=49) 60.5%	40.8%	c2=160.676 d.f.=1	(n=65) 40.1%
Minority	(n=65) 80.2%	89.1%	*p*<0.0001	(n=32) 39.5%	59.2%	*p*<0.0001	(n=97) 59.9%
Hispanic	(n=56) 69.1%	69.2%		(n=20) 24.6%	25.5%		(n=76) 46.9%
Black	(n=5) 6.2%	9.6%		(n=7) 8.6%	12.2%		(n=12) 7.4%
Asian/Other	(n=4) 4.9%	10.3%		(n=5) 6.2%	1.9%		(n=9) 5.6%

Table 2. DNA isolation and recovery

	DNA recovery	Unsuccessful	Analysis/Recovery
Pediatric samples	n=76	n=5	93.8% (n=76/81)
			ave.= 171.29 ng/uL
			A260/A280: 1.47-2.0
	n=76; GAPDH		
	n=76; 16S rRNA		
Adult samples	n=73	n=8	90.1% (n=73/81)
			ave.= 218.3 ng/uL
			A260/A280: 1.55-2.0
	n=73; GAPDH		
	n=73; 16S rRNA		

Table 3. Analysis of PCR screening

	SW-positive	SW-negative	Statistics
Pediatric samples	n=20 (26.3%)	n=56 (73.7%)	c^2=0.502, d.f.=1
Adult samples	n=18 (24.7%)	n=55 (75.3%)	*p*=0.4786
Combined	n=38 (25.5%)	n=111 (74.5%)	
	SM-positive	SM-negative	Statistics
Pediatric samples	n=36 (47.4%)	n=40 (52.6%)	c^2=7.475, d.f.=1
Adult samples	n=41 (56.2%)	n=32 (43.8%)	*p*=0.0063
Combined	n=77 (51.7%)	n=72 (48.3%)	
	PG-positive	PG-negative	Statistics
Pediatric samples	n=12 (15.8%)	n=64 (84.2%)	c^2=19.121, d.f.=1
Adult samples	n=18 (24.7%)	n=55 (75.3%)	*p*<0.001
Combined	n=30 (20.1%)	n=119 (79.9%)	

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Figure 1. DNA standards and Semi-Quantitative Analysis. Standard curves using PCR band intensity at 30 cycles (C30) from serial dilutions of cells at 5.0 x 10^6, 10^5, and 10^4 cells (HGF-1) or CFU/mL (*S. mutans*, *P. gingivalis*) demonstrate signal band intensity (SBI) correlated strongly with starting cell number (R^2 range: 0.9879-0.9947), thus providing a semi-quantitative method for estimating cell number/mL.

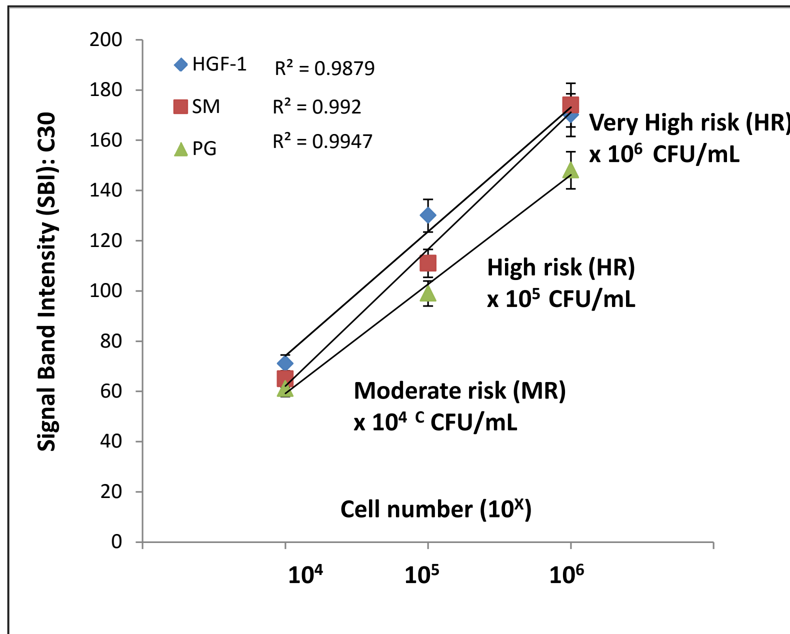

Figure 2. Semi-quantiative assessment of CFU/mL and disease risk. Categorization of signal band intensity (SBI) at C30 allowed for the estimation of bacterial levels in saliva samples using CFU/mL. Most samples were below the limit of detection (bLOD) or in the normal or "average" low-risk (LR) categories. Fewer samples were found to harbor moderate (MR), high (HR) or very high (vHR) levels of microbial pathogens.

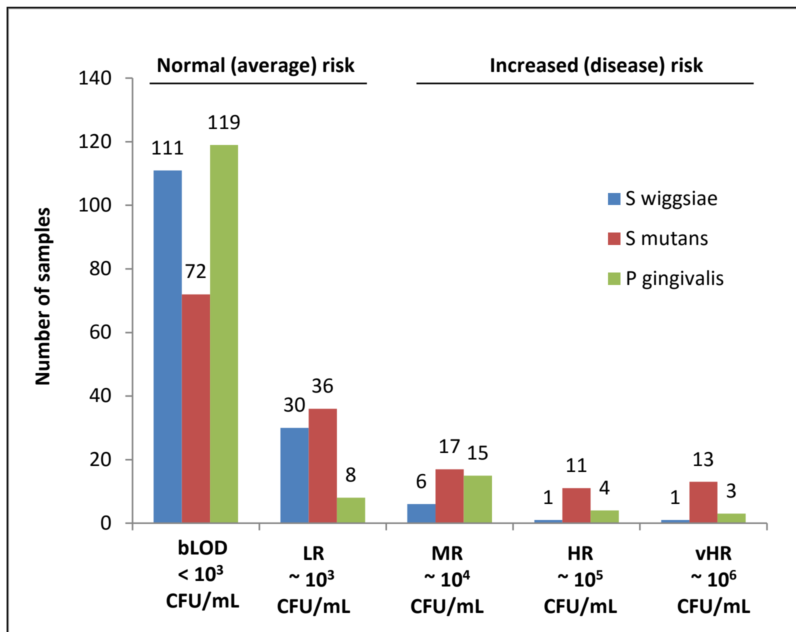

Table 4. Analysis of correlation with demographic and health parameters.

	Pediatric samples	Adult samples	Correlation
DMFT score (ave.)	5.82	22.95	R=-0.203 (low)
BMI (ave.)	23.48	26.9	R=0.131 (low)
Age (ave.)	9.1	43.75	R=0.143 (low)
Race	N/A	N/A	R=0.191 (low)
Gender	N/A	N/A	R=0.137 (low)

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Finally, the association of health parameters and other demographic information was compared with SW-positivity. This analysis revealed an inverse correlation with decayed, missing and filled teeth (DMFT) score average, which was lower among pediatric patients (5.82) than adults (22.95), R=-0.203. However, no statistically significant associations were found using body mass index (BMI), age, race, or gender.

DISCUSSION

The overall purpose of this study was to analyze the prevalence of *Scardovia* from a prospective sampling of pediatric and adult clinic populations at a public dental school. These results demonstrated that a remarkable 25.5% of samples had detectable levels of *Scardovia*, which is much higher than the results of a similar (but smaller) retrospective analysis (4.9%) from this patient population.[10] Although the overall percentage of samples above the limit of detection (LOD) was much greater, the semi-quantitative analysis approximating cell number suggested that most of these samples were in the low risk (LR) category, which was similar to the previous findings. These results strongly suggest that this newly identified cariogenic pathogen is present within saliva samples from the patient population, which may further enhance our understanding of the microbial constituents that comprise the oral biofilm and may contribute to dysbiosis.[4,5]

These results also appear to confirm previous reports that suggest *S. mutans* may be the dominant cariogenic pathogen within the oral cavity.[4-6] However, these data also demonstrated that although *Scardovia* was present in nearly half of samples that also tested positive for *S. mutans*, many samples with average or undetectable levels of *S. mutans* also harbored low to moderate levels of *Scardovia*. These data may therefore suggest that at least a subset of patients may harbor vastly different compositions of microbial pathogens that may influence their caries risk over time.

In addition, the concomitant collection of health and demographic data was possible due to the prospective nature of this study. However, these analyses revealed few associations with any statistical significance – which further complicates our understanding of these phenomena. For example, although there was a negative or inverse correlation with DMFT score, this may be explained by the fact that older patients typically have higher DMFT scores.[18,19] This conclusion appears to be supported by the finding that age was not significantly associated with *Scardovia* detection, which may suggest other factors may be more important in determining which patients may be at higher risk.

Other potential factors that have been found to predict caries risk and caries lesions among this population include race and BMI.[20,21] However, the results of this study suggested that neither race nor BMI were significantly associated with *Scardovia* detection, which provides further evidence that the typical risk factors associated with cariogenic bacteria may not be uniformly distributed among patients harboring this organism.

Although this study was among the first prospective clinic screenings, there are several limitations which should be considered when evaluating this evidence. For example, although the sample size was comparable to other studies of this nature, the overall percentage of clinic patients that have been screened remains low (<1%). In addition, a complete understanding of the microbial interactions that may inhibit or support the growth of *Scardovia* within the oral cavity remains unknown. Finally, until culturing and growth conditions are standardized and samples become widely available, many other confounding variables in diet and nutrition may remain impossible to evaluate.

CONCLUSIONS

This study confirms previous reports of a newly identified cariogenic pathogen, *Scardovia wiggsiae*, within a dental school patient population. The current findings suggest that nearly one quarter of patients may harbor this organism, although the factors that influence whether this bacterial is present remain unknown. Many of the traditional risk factors for caries, including DMFT score and BMI do not appear to be associated with positive screening results, which suggest more research may be needed to fully elucidate the long-term risk and the demographic or health parameters associated with this organism.

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