Oral Site Specific Sampling Reveals Differential Location for *Scardovia wiggsiae*

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Authors’ contributions

This work was carried out in collaboration among all authors. Authors AA, LN and GC were involved in data collection and sample preparation. Authors GC and KK were responsible for overall study design and data analysis. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: The newly discovered cariogenic pathogen *Scardovia wiggsiae* has been the subject of intense scientific interest due to the role it may play in the development or progression of caries and oral disease. The primary objective of this study was to perform DNA microbial screening from five specific oral sites, including the gingival crevice between the upper central incisors, biofilm of the upper first molar and lingual incisor, as well as the dorsum of the tongue — for comparison with unstimulated saliva. These data may provide significant insights into site-specific oral locations that harbor *S. wiggsiae*.

Methods: More than one hundred previously collected clinical samples (n=105) were identified for inclusion in this study. DNA isolates were screened using a NanoDrop spectrophotometer to
determine overall DNA quantity and quality. Samples with sufficient quality and quantity were screened for the presence of \textit{S. wiggsiae} using validated PCR primers.

**Results:** More than one hundred patient samples (n=105) were identified, which were comprised of mostly female (57%) versus male (43%) and minority (71%) versus White (29%). The average DNA concentrations ranged between 13.74 and 14.69 \(\mu\text{g/\muL}\), with A260:A280 ratios ranging between 1.62 – 1.70. Results of molecular screening using \textit{S. wiggsiae} specific primers demonstrated only a small percentage of pooled samples (7.6%) harbored this DNA, which was highly concentrated among the samples from tooth surfaces (Upper First Molar, Lingual Incisor) and saliva compared with the gingival crevice and dorsum of the tongue.

**Discussion:** These data provide novel information regarding specific oral locations, including tooth surfaces that harbor \textit{S. wiggsiae}. In addition, these sites also provide new information regarding oral sites that do not appear to harbor this organism, including the gingival crevice and dorsum of the tongue. This information may be particularly useful to oral health researchers as they strive to limit and reduce the cariogenic microbiome among high-risk populations.

**Keywords:** Scardovia wiggsiae; caries; pathogen; screening.

**ABBREVIATIONS**

Severe early childhood caries (SECC), Early Childhood Caries (ECC), Institutional Review Board (IRB), Office for the Protection of Research Subjects (OPRS), University of Nevada, Las Vegas (UNLV), School of Dental Medicine (SDM), Paper Points (PP), Phosphate Buffered Saline (PBS), biosafety level (BSL-2), Relative Centrifugal Force (RCF), Melting Temperatures (Tm).

1. **INTRODUCTION**

The oral microbiome is comprised of a rich and complex network of organisms that play significant roles in the maintenance of good oral health but also in the development of oral disease, such as dental caries [1,2]. The newly discovered cariogenic pathogen \textit{S. wiggsiae} has been the subject of intense scientific interest due to the role it may play in the development or progression of caries and oral disease [3,4]. \textit{Scardovia} was of interest in oral health research for the significant contributions to severe early childhood caries (SECC), both in the presence and absence of canonical cariogenic organisms – such as \textit{S. mutans} [5]. This gram-positive anaerobic bacillus is both acidogenic and acid tolerant, which are known to be the most significant contributing virulence factors towards the development of dental caries [6].

More evidence is now emerging regarding the prevalence and epidemiology of \textit{Scardovia wiggsiae}, particularly among children with early childhood caries (ECC) [7,8]. These studies clearly describe the potential for caries development and pathology among children and teenagers both in the presence and absence of other clearly defined cariogenic organisms, such as \textit{Streptococcus mutans} [9-11]. However, less is known about the prevalence and epidemiology of this organism among adult populations and populations without significant caries experience [12,13].

Recent studies from this group have begun to elucidate the prevalence and epidemiology of \textit{Scardovia} among both pediatric and adult populations, with and without caries experience [14-16]. Although these data have provided evidence of \textit{Scardovia} in both pediatric and adult populations with and without caries experience, more detailed epidemiology of this organism among high-risk groups including Orthodontic patients is continuing [17-19]. Many of these studies have screened unstimulated saliva using highly specific molecular techniques, but few studies to date evaluated the presence of this organism at specific sites within the oral cavity [11,20].

If recommendations are to be made in order to improve oral health and reduce risk for disease caused by this organism, a more specific oral microbial sampling must be completed to determine if methods such as flossing (specific to improve gingival health and focused on the gingival crevice) or brushing (more targeted towards supragingival plaque and biofilm) might be more effective at disease prevention [13]. The primary objective of this study was to perform DNA microbial screening from five specific oral
sites that were previously derived using sterile paper points, including the gingival crevice between the upper central incisors, biofilm of the upper first molar and lingual incisor, as well as the dorsum of the tongue— for comparison with unstimulated saliva. These data may provide significant insights into site-specific oral locations that harbor S. wiggsiae.

2. MATERIALS AND METHODS

2.1 Human Subjects

The original protocol for sample collection was reviewed and approved by the Institutional Review Board (IRB) in the Office for the Protection of Research Subjects (OPRS) at the University of Nevada, Las Vegas (UNLV) titled “The Prevalence of Oral Microbes in Saliva from the University of Nevada, Las Vegas — School of Dental Medicine pediatric and adult clinical population” (OPRS#1502-506M). Briefly, inclusion criteria were any UNLV School of Dental Medicine (SDM) clinic patient that agreed to participate. Exclusion criteria were any UNLV-SDM patient (or parent / guardian of patients under the age of 18 years) that declined to participate and any person not a patient at a UNLV-SDM clinic. No patients received money or services in exchange for participation. All patients that volunteered for the original study asked provided Informed Consent (and Pediatric Assent if under the age of 18 years old).

2.2 Clinical Samples

In brief, saliva collection from the original protocol was facilitated using a sterile sample collection container (50 mL conical centrifuge tube) with patients providing up to 5.0 mL of unstimulated saliva. During the clinical oral exam, site-specific oral sampling was performed using sterile paper points (PP) to collect from the gingival crevice between the front incisor (Tooth 9), the buccal surface of an upper maxillary molar (Tooth 3), the lingual surface of a mandibular incisor (Tooth 25), as well as the dorsum of the tongue. Each paper point was placed into isotonic 1X phosphate buffered saline (PBS) solution and stored on ice prior to transfer to a biomedical biosafety level (BSL-2) laboratory for analysis. Each patient sample was given a randomly generated, non-duplicated number for laboratory analysis, which was not linked to any patient information or other identifying information. Only patient age, sex and ethnicity were noted for subsequent demographic analysis.

2.3 Sample Processing

All clinical samples were processed to isolate DNA using the Invitrogen TRIzol reagent and protocol, which has been approved to process liquid and viscous clinical samples (blood, semen, saliva, sputum) to obtain DNA of sufficient quality for polymerase chain reaction (PCR) screening [14,21]. Briefly, PP samples were vortexed for 20 – 30 seconds to remove any adsorbent bacteria. The TRIzol reagent was added to 1000 µL of the saliva or PP eluted samples and incubated prior to the addition of chloroform. Samples were then centrifuged at 12,000 g or relative centrifugal force (RCF) to isolate the nucleic acids (upper aqueous phase) from the solids and other proteins. Ethanol (100%) was added to each DNA isolate to facilitate precipitations and pellets were then centrifuged at 2,000 g or RCF and washed with 75% ethanol prior to resuspension in 100 µL of DNA rehydration solution. Quality and quantity of DNA was measured using a NanoDrop spectrophotometer at absorbance readings of A260 and A280.

2.4 PCR Screening

Molecular screening for the presence of S. wiggsiae was accomplished using PCR with the following reaction parameters: Initial incubation at 50°C x 2 minutes, Denaturation at 95°C x 10 minutes and 30 cycles at the annealing (melting) temperatures (Tm) indicated below using primers synthesized from Eurofins MWG Operon:

**Positive control**

16s rRNA bacterial primer set

Forward 5'-ACG CGT CGA CAG ACT TTG ATC CTG GCT-3'; 27 nt; 56% GC; Tm: 76°C

Reverse 5'-GGG ACT ACC AGG GTA TCT AAT-3'; 21 nt; 48% GC; Tm: 62°C

**Scardovia wiggsiae primer set**

Forward 5'- GTG GAC TTT ATG AAT AAG C-3'; 19 nt; 37% GC; Tm: 55°C

Reverse 5'- CTA CCG TTA AGC AGT AAG-3'; 18 nt; 44% GC; Tm: 56°C
Scardovia wiggsiae Optimal temperature for primer set: Lower temperature – 5°C = 50°C.

2.5 Statistical Analysis

Analysis of patient demographics was presented as simple descriptive statistics. Any differences between the study sample and the overall clinic demographics were assessed using Chi Square (χ²), which was appropriate for non-parametric data analysis [22]. Analysis of screening results is also presented as descriptive statistics.

3. RESULTS

More than one hundred patient samples (n=105) were identified for inclusion in this analysis (Table 1). The majority of these samples were originally derived from female patients (57%), which closely resembled the overall clinic population (60%), p=0.543. The racial and ethnic composition of the study samples was primarily from non-White minority patients (71.5%), which reflected the composition of the clinic population (75%), p=0.3556. In addition, roughly half of the samples were derived from pediatric patients (52%), which was also similar to the composition of the clinic from which they were derived (57%), p=0.3125.

To evaluate whether the DNA isolated from these samples was appropriate for molecular screening, absorbance readings at A260 and A280 nm were combined to provide estimates of DNA quantity and quality (Table 2). The average DNA concentration from each of the oral sampling sites was not significantly different from the average DNA concentration obtained from whole, unstimulated saliva (13.74 µg/µL), p=0.7892. Although significant ranges in DNA concentration were observed between different patients, DNA concentrations from different oral sites within the same patient were not, p=0.6979. Measurement of DNA quality using the absorbance ratio A260:A280 demonstrated sufficient quality for all samples using the PCR screening (>1.55), ranging between 1.62–1.70.

Each sample was then screened using the positive control primers for bacterial DNA, 16S rRNA (Fig. 1). These data demonstrated that all samples screened produced PCR bands with signal band intensity (SBI) greater than the limit of detection (LOD). Graphical analysis of PCR screening results demonstrated no specific patterns between 16S rRNA SBI and specific oral sites could be determined.

Due to the low prevalence of Scardovia observed in previous studies [14-19], more efficient screening was facilitated by pooling DNA isolates from each patient together (PP: Gingival crevice, PP: Upper first molar, PP: Lingual incisor, PP: Tongue dorsum; Saliva) (Fig. 2). Using an equal volume of DNA from each site, the combined pooled samples were comprised of approximately 15-20% of the total sample from each site (Fig. 2A). Screening of the pooled samples using the S. wiggsiae specific primers revealed only a small percentage of pooled samples (7.6%) generated positive PCR screening results (Fig. 2B).

Each of the corresponding site specific samples that comprised the Scardovia PCR-positive pooled samples were then screened separately (Fig. 3). This analysis revealed that each of oral sites from the pooled samples tested were found to harbor S. wiggsiae, although much stronger signal band intensities were observed among the PP samples from supragingival plaque or biofilm from tooth surfaces (Upper First Molar, Lingual Incisor) and saliva.

| Table 1. Demographic analysis of study sample |
|-----------------------------------------------|
| Study sample | Clinic population | Statistical analysis |
| Sex          |                  |                      |
| Female       | 57.1%            | 60.4%                | χ²=0.375 |
| Male         | 42.9%            | 39.6%                | d.f.=1   |
|              |                   | p=0.5403             |          |
| Race / Ethnicity |                  |                      |
| White        | 28.5%            | 24.7%                | χ²=0.853 |
| Minority     | 71.5%            | 75.3%                | d.f.=1   |
|              |                   | p=0.3556             |          |
| Age          |                  |                      |
| Pediatric (<18 years) | 52.3% | 56.7% | χ²=1.020 |
| Adult (>18 years)     | 47.7%            | 43.3%                | d.f.=1   |
|                      |                   | p=0.3125             |          |
Fig. 1. 16S rRNA screening of patient samples. Molecular screening of patient samples using PCR revealed 16S rRNA expression determined by signal band intensity (SBI) greater than the limit of detection (LOD) with no specific patterns observed between SBI and specific oral sites.

Fig. 2. Pooled DNA from clinical samples screened for 16S rRNA. A) Pooling of DNA from each oral site (15-25%) for each individual patient created an efficient screening process. B) Results of molecular screening using *S. wiggsiae* specific primers demonstrated only a small percentage of pooled samples (7.6%) harbored this DNA.
Table 2. Analysis of DNA concentration and purity from study sample

|                          | DNA concentration | DNA quality (A260:A280) | Statistical analysis |
|--------------------------|-------------------|-------------------------|----------------------|
| Saliva (whole)           | 13.74 ug/uL (ave.)| 1.62                    |                      |
|                          | 4.41 – 46.1 ug/uL (range) | 1.38 – 2.03           |                      |
| Gingival crevice (PP)    | 14.69 ug/uL (ave.)| 1.70                    | Two tailed t-test    |
|                          | 4.76 – 48.8 ug/uL (range) | 1.46 – 2.12           | p=0.7892             |
| Dorsal tongue (PP)       | 14.34 ug/uL (ave.)| 1.64                    | Two tailed t-test    |
|                          | 6.1 – 48.2 ug/uL (range) | 1.37 – 2.11           | p=0.8527             |
| Lingual incisor (PP)     | 14.4 ug/uL (ave.) | 1.67                    | Two tailed t-test    |
|                          | 4.3 – 48.6 ug/uL (range) | 1.33 – 2.16           | p=0.8458             |
| Upper first molar (PP)   | 14.3 ug/uL (ave.) | 1.69                    | Two tailed t-test    |
|                          | 4.1 – 48.7 ug/uL (range) | 1.44 – 2.11           | p=0.8608             |

Fig. 3. PCR screening of site specific samples from corresponding Scardovia PCR-positive pooled samples. Each of oral sites from the pooled samples tested harbored S. wiggsiae, with stronger signal band intensities observed among the PP samples from tooth surfaces (Upper First Molar, Lingual Incisor) and saliva than the gingival crevice or dorsum of the tongue.

4. DISCUSSION

The primary objective of this study was to perform DNA microbial screening from five specific oral sites including the gingival crevice between the upper central incisors, biofilm of the upper first molar and lingual incisor, as well as the dorsum of the tongue – for comparison with unstimulated saliva. These data have revealed significant insights into site-specific oral locations that harbored S. wiggsiae. For example, although previous studies have identified Scardovia from caries-specific lesions and from whole saliva – this may be among the first studies to evaluate and screen for this pathogen from additional oral sites from patients without significant caries experience [3,5,8].

These data suggest that mandibular and maxillary surfaces from both anterior and...
posterior sites may be preferential oral locations, which may be significant as new evidence has now emerged that has suggested biomaterials and bioactive materials may selectively inhibit the virulence and modulate the microbial ecology of biofilms that include this organism [23,24]. Combining these agents with these data regarding oral location may be particularly useful for oral health researchers interested in selectively placing these agents among high risk populations, such as children with SECC [1,4,7]. These data may also be useful towards understanding the location and balance of organisms that comprise the caries microbiome in an effort to improve prevention and treatment strategies for children and young adults [25,26].

Despite the significance of these findings, some limitations inherent to this type of study should also be considered when evaluating these results. For instance, this study involved analysis of saliva samples from a predominantly low-income, minority-serving public dental school clinic [27-29]. This may suggest this sample set may have a lower health literacy and higher risk for caries than a random sampling of the overall population. This type of sampling bias could have influenced the findings and results of this study in ways that are not easy to predict. In addition, due to financial and other funding constraints, only a limited number of samples could be collected and analyzed for this project – which may also place some limitations on the overall generalizability of these results.

5. CONCLUSIONS

Although this study has some limitations due to the study population and sample size, these data provide novel information regarding specific oral locations, including tooth surfaces that harbor S. wiggsiae. In addition, these sites also provide new information regarding oral sites that do not appear to harbor this organism, including the gingival crevice and dorsum of the tongue. This information may be particularly useful to oral health researchers as they strive to limit and reduce the cariogenic microbiome among high-risk populations.

CONSENT

All patients volunteered for the original study provided informed and written consent (Pediatric Assent if under the age of 18 years old).

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the author(s).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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