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Screening for the Novel Cariogenic Pathogen Scardovia Wiggsiae among Orthodontic Patients

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SCREENING FOR THE NOVEL CARIOGENIC PATHOGEN SCARDOVIA WIGGSIAE AMONG ORTHODONTIC PATIENTS

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Abstract

Screening for the novel cariogenic pathogen Scardovia wiggsiae among Orthodontic patients

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The recent discovery of a novel cariogenic pathogen Scardovia wiggsiae has led many scientists and oral health researchers to re-evaluate and re-examine existing saliva repositories to determine the prevalence among patient populations. Recent efforts at this institution have used existing saliva samples to determine the prevalence among both adults and pediatric patients. These studies have revealed this organism may be found in approximately one-quarter of all samples tested.

However, the introduction of orthodontic brackets has traditionally increased the risk of caries lesions and the growth of cariogenic organisms – which may suggest the prevalence of this organism may be different among orthodontic patient populations. To determine if any differences could be found among the adult and pediatric orthodontic patient population, retrospective screenings of previously collected orthodontic patient saliva were performed. These studies revealed a similar but slightly lower prevalence among adult orthodontic patients but a much higher (almost twice) prevalence among pediatric orthodontic patients.

Although these data have been generated from retrospective analysis of existing saliva repositories, the results to date strongly suggest an inverse, age-dependent relationship between orthodontic treatment and Scardovia prevalence. Higher percentages of pediatric (younger) orthodontic patients in both studies harbored this organism, while no similar finding was observed among adult orthodontic patients

Due to the recent discovery of Scardovia wiggsiae, few studies have gathered sufficient information to provide information regarding prevalence – particularly among high-risk populations. The combined data from each of the five studies at this institution provide strong evidence that prevalence is similar among pediatric and adult populations, however those pediatric patients undergoing orthodontic therapy and treatment may exhibit much higher prevalence of this organism for reasons that have yet to be elucidated. More research will be needed to discovery the underlying reasons for these findings and to determine if the presence (or absence) of this organism may be related to higher or lower caries risk.

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Chapter 1: Introduction

Background and Significance

Cariogenic bacteria are an important focus of research as almost 50% of children and most adults in the United States are affected by dental caries [1]. The prevalence of recently discovered Scardovia wiggsiae (SW) within the UNLV School of Dental Medicine (UNLV SDM) patient population has been analyzed in previous studies [1 -3]. SW has also been found be significantly associated with early childhood caries, a condition affecting 28% of children in the United States [4, 5]. Although these studies have been conducted to study its prevalence in both pediatric and adult populations, the prevalence of SW has not been evaluated among pediatric patients who have orthodontic appliances.

Orthodontic appliances increase the risk of developing white spot lesions, an early stage of carious lesion development [6]. A major reason behind the increased development of pre-carious and carious lesions is due to increased difficulty in effectively removing plaque on all tooth surfaces when orthodontic appliances are present. Fixed orthodontic appliances may also prevent the oral environment's innate ability to cleanse itself with salivary flow and soft tissue movements. Plaque is one of the major niches for cariogenic bacterial growth and will contribute to the formation of white spot lesions, and eventually carious lesions [7, 8]. Having a firm understanding of how the oral environment is changed during orthodontic treatment is imperative in developing strategies to ensure the risk to patients during orthodontic treatment is minimized as much as possible. This would allow for healthier, more predictable treatment results if prevalence of cariogenic bacteria could be managed.

The primary focus of this study will be comparison of SW prevalence among the pediatric orthodontic patient samples for comparison with samples taken from an age-matched cohort of non-orthodontic patients. Furthermore, the study will also include comparison between pediatric and adult patients with orthodontic appliances.

Methods and Materials

A retrospective analysis of previously collected saliva samples from pediatric orthodontic patients will be used for comparison with age-matched samples from non-orthodontic patients, as well as adults with orthodontic appliances. Samples from the previous study (Protocol OPRS#1305-4466M: The Prevalence of Oral Microbes in Saliva from the UNLV School of Dental Medicine pediatric and adult clinical population) approved May 22, 2013 will be used (n=408). In brief, patients from the pediatric, orthodontic, and general UNLV-SDM clinics were asked to participate in the study. Subjects who agreed to participate were given a small, sterile saliva collection container, 50 mL sterile polypropylene tube (Fisher Scientific: Fair Lawn, New Jersey, USA) and asked to spit into it for a full minute. Samples were stored on ice until transport to a biomedical laboratory for analysis. Each saliva sample was assigned a unique, randomly-generated number to prevent research bias. On all subjects the following data was collected concurrently; gender, race/ethnicity, age, and number of decayed missing or filled teeth (DMFT).

For this project, samples will be sorted into Pediatric (< 18) and Adult (> 18) categories and then further separated into orthodontic and non-orthodontic patients. DNA will be isolated from these samples and will subsequently be screened for SW using polymerase chain reaction (PCR) and

primers specifically designed to distinguish this organism [9]. Results from the pediatric orthodontic patients will be compared to those from non-orthodontic patients and will be analyzed for any significance in presence based on gender, race/ethnicity, age, and number of decayed missing or filled teeth (DMFT), as well as other relevant health parameters. Recent literature analyzing SW prevalence in adult populations will be utilized when comparing SW prevalence in pediatric populations [2].

Research Questions

1. Does the prevalence of S. wiggsiae vary between orthodontic and non-orthodontic pediatric patients?
    a. H_0: Orthodontic and non-orthodontic pediatric patients will have similar prevalence of S. wiggsiae.
    b. H_a: Orthodontic and non-orthodontic pediatric patients will have differences in S. wiggsiae.
2. Does the prevalence of S. wiggsiae vary between adult and pediatric orthodontic patients?
    a. H_0: Adult and pediatric orthodontic patients will have similar prevalence of S. wiggsiae.
    b. H_a: Adult and pediatric orthodontic patients will have differences in S. wiggsiae.

Research Design

The primary research design of this study will be retrospective and observational in nature. Only existing saliva samples collected in UNLV clinics will be analyzed in the study. Since no new samples are to be collected, a request for an IRB exemption will be filed. The main outcome variable will consist of a binary PCR screening result: positive (+) or negative (-); Additional information can be evaluated regarding relative levels (CFU/mL of saliva). The main predictor variable will consist of Orthodontic treatment. The confounding variables will consist of demographic variables including age, gender, race/ethnicity, and some basic clinical and health information (BMI, oral lesions, DMFT score).

Statistical Analysis

Because the difference in prevalence between groups (Orthodontic, non-Orthodontic) are to be measured from a cross-section of samples taken from a cohort or convenience sample, a preliminary analysis using a two-tailed t test can be reasonably employed to discern any statistical difference. As long as the sample size is at least moderate from each group (~20), quite severe departures from normality make little practical difference in the conclusions reached from these analyses. In addition with a sample size of (~20) a chi-square can easily be used to discern any statistical correlation between prevalence and age of the patient.

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Chapter 2

Cariogenic pathogen Scardovia wiggsiae screening among pediatric orthodontic patients: A pilot study

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Role of Authors:
Dr. Adam Whiteley designed the study and worked with dental students Weston Milne and Ghazaleh Rezaei for data generation and collection. Dr. Karl Kingsley was secondary author and assisted Dr. Whiteley with data analysis.

Abstract

Background: Dental caries remains one of the most prevalent oral health diseases in the United States, affecting nearly half of all children and a majority of adults. Most medically important cariogenic bacteria, including *Streptococcus*, *Lactobacillus*, *Actinomyces* and *Veillonella* species are well known, although recent evidence has identified the new cariogenic pathogen *Scardovia wiggsiae* (*S. wiggsiae*) among children and minorities with severe early childhood caries. Based upon these new findings, the goal of this project was to determine the prevalence of this new cariogenic pathogen *S. wiggsiae* from a repository of previously collected pediatric saliva samples from orthodontic patients. Methods: DNA was isolated from previously collected saliva samples (n=48) and was subsequently screened for the presence of *S. wiggsiae* using polymerase chain reaction (PCR) and primers designed specifically to distinguish this organism. Results: Fifteen (15) samples tested positive for *S. wiggsiae*, representing 31.25% of the samples screened. Conclusions: As previous studies from this laboratory using adult orthodontic patients and pediatric non-orthodontic patients revealed prevalence of and 14% and 21.5%, respectively -

these findings suggest that the newly identified cariogenic pathogen *S. wiggsiae* may disproportionately affect pediatric orthodontic patients for reasons that are not well understood, which imply more detailed and focused research in this area is needed. As previous research has demonstrated that oral health status and caries risk may be related to education, income, and socioeconomic status, these findings help to elucidate and contextualize the risks facing these populations.

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

Background

Dental caries remains a big problem in the world and particularly in developed countries [1]. Despite the advances in oral health care products and services, there are many forces that may influence the rate and distribution of dental caries, especially among children [2,3]. For example, the increased prevalence of sugar sweetened beverages, poor or non-existent dietary education, and lack of dental health insurance have conspired to create a problem even among affluent societies [4,5].

Orthodontic treatment has increased in popularity in Western countries – and is almost routine or commonplace in the US among teenagers and adolescents [6,7]. Orthodontic brackets remain the most widely used form of treatment, which can be associated with decreased oral hygiene and increased risk of oral caries [8,9]. The most detailed research studies have focused necessarily on the most widely accepted cariogenic pathogens, including *Streptococcus mutans* as well as *Lactobacillus*, *Actinomyces*, and *Veillonella*.

More recent studies, however, have demonstrated that other cariogenic pathogens may also be present and are now known to contribute significantly to dental caries [12,13]. This includes *Scardovia wiggsiae*, which was originally isolated from pediatric patients with severe early childhood caries but has more recently been found among other patients [14-16]. Some studies have even found *S. wiggsiae* among adult orthodontic patients, thereby highlighting the need to further study prevalence among pediatric orthodontic patients [17,18].

Due to the increased caries risk associated with orthodontic treatment in general, and in pediatric patients more specifically, the goal of this study was to use an existing saliva repository to identify any pediatric orthodontic patient samples that could be screened for *Scardovia wiggisae*.

Methods

Human subjects

Approval for this retrospective study of previously collected saliva samples titled "Retrospective investigation of Prevalence of *Scardovia wiggsiae* (SW) in pediatric orthodontic patients " (Protocol#880427-1) was granted by the UNLV Office for the Protection of Research Subjects (OPRS) Institutional Review Board (IRB) on March 7, 2016. The original protocol for the collection of saliva samples titled "The Prevalence of Oral Microbes in Saliva from the University of Nevada Las Vegas (UNLV) School of Dental Medicine (SDM) pediatric and adult clinical population" (Protocol#1502-5068M) was reviewed and approved by the UNLV Office for the Protection of Research Subjects (OPRS) Institutional Review Board (IRB) on February 6, 2015.

In brief, parents or guardians were asked to participate in this study and Informed Consent was obtained. Pediatric patients were then asked for their voluntary participation and Pediatric Assent was also obtained. Participation was strictly voluntary and no remuneration was given to any subject. Patients were given a sterile saliva collection tube and asked to provide up to 5 mL of unstimulated saliva. Samples were then transferred to a biomedical laboratory for analysis.

DNA isolation

The isolation of DNA from saliva samples was performed as previously described [19,20]. In brief, samples were processed using the GenomicPrep DNA isolation kit from Amersham Biosciences (Buckinghamshire, UK) using the manufacturer recommended protocol. The isolated DNA was suspended in 100 uL of DNA hydration solution for quality and quantity analysis using absorbance ratio measurements at A260 and A280 nm.

PCR screening

Polymerase Chain Reaction (PCR) screening was performed using the Fisher Scientific exACTGene complete PCR kit (Fair Lawn, NJ) and the Eppendorf Mastercycler (Hamburg, Germany), as previously described [16,18]. The PCR positive control used to confirm the presence of human DNA from saliva samples was glyceraldehyde- 3- phosphate dehydrogenase (GAPDH) and the PCR positive control for the presence of bacterial DNA within each saliva sample was the 16S rRNA universal primer. Screening for the cariogenic pathogen *Scardovia wiggsiae* was then accomplished using the following primers [13,17].

GAPDH forward primer, ATCTTCCAGGAGCGAGATCC; Tm=66°C

GAPDH reverse primer, ACCACTGACACGTTGGCAGT; Tm=70°C

16S rRNA universal primer, ACGCGTCGACAGAGTTTGATCCTGGCT; Tm=76°C

16S rRNA universal primer, GGGACTACCAGGGTATCTAAT; Tm=62°C

S. wiggsiae forward primer, GTGGACTTTATGAATAAGC; Tm=55°C

S. wiggsiae reverse primer, CTACCGTTAAGCAGTAAG; Tm=56°C

In brief, each PCR reaction was performed using one ug of total DNA. The initial denaturation step ran for three minutes at 94°C, with a total of 30 amplification cycles (C30) consisting of 30 second denaturation at 94°C, 60 seconds of annealing at 55°C for *S. wiggsiae*, 66°C for GAPDH and 62°C for 16S, 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).

Statistical analysis

Demographic data for the study sample are presented as absolute number (n=X) and using descriptive statistics (percentage or %), which were compared to the clinic population using Chi-square analysis from GraphPad software (La Jolla, CA). Statistical significance was denoted as $p<0.05$.

Results

Demographic analysis of the retrospective samples identified was performed (Table 1). This

analysis revealed that the percentage of females and males within the study sample (52% and 48%, respectively) was not significantly different from the overall composition of the clinic population (49% and 51%, p=0.0577). The reported racial and ethnic background of the study sample isolates was also similar to the overall clinic population with approximately 2/5 of the sample White and 3/5 of the sample from non-White or minority backgrounds (p=0.8473). The study sample contained only pediatric orthodontic patients averaging 16.6 years of age, while the overall orthodontic clinic population is comprised of both pediatric and adult populations, with an average age of pediatric orthodontic patients equal to 15.8 years.

Table 3. Demographic analysis of study sample.

	Study sample (n=48)	Clinic population	Statistical analysis
Sex			$\chi 2$=3.601
Female	52.15% (n=25)	49.1%	d.f.=1
Male	47.9% (n=23)	50.9%	p=0.0577
Race/Ethnicity			$\chi 2$=0.037
White	41.7% (n=20)	41.4%	d.f.=1
Minority	58.3% (n=28)	58.6%	p=0.8473
Hispanic	35.4% (n=17)	35.9%	
Black	18.8% (n=9)	13.1%	
Asian	4.2% (n=2)	4.2%	

Age		
12-17 years	Ave.=16.6yrs +/-1.4	15.8 yrs. +/- 3.2
18+ years		21.4 yrs. +/- 2.4
	Range (11-17 yrs.)	Range (11-38)

The pediatric orthodontic saliva samples that were identified from the existing repository were then processed to isolate DNA contained within the sample, including bacterial and human DNA (Table 2). DNA was successfully isolated from all study samples (n=48) with an average concentration of 261.3 ng/uL, which is within the acceptable range provided by the manufacturer. The purity of each sample was determined using the ratio of absorbance measurements at A260 nm and A280 nm, which ranged between 1.62 and 2.00 with an average of 1.74 - which allowed for the subsequent screening of all identified samples using PCR.

Table 4. DNA isolation and analysis

	DNA recovery	**Quantification**	**Purity**
Study samples	n=48	261.3 ng/uL	A260:A280
	(100%)	+/- 63.1 (STD)	1.62 – 2.00
			Ave=1.74
Manufacturer range	95-100%	100-1000 ng/uL	1.50 – 2.00
			Purity 1.70 – 2.00

The isolates from each of the saliva samples were then screened using PCR for the positive control genes for human (GAPDH) and bacterial (16S rRNA) DNA (Figure 1). The PCR results for GAPDH revealed that each sample had detectable human DNA (n=48), while similar positive results were found for 16S rRNA (bacterial DNA, n=48), confirmation that all samples contained both human and bacterial DNA. The PCR screening results for *S. wiggsiae* revealed that approximately one-third (n=15/48 or 31.25%) of these isolates harbored this organism.

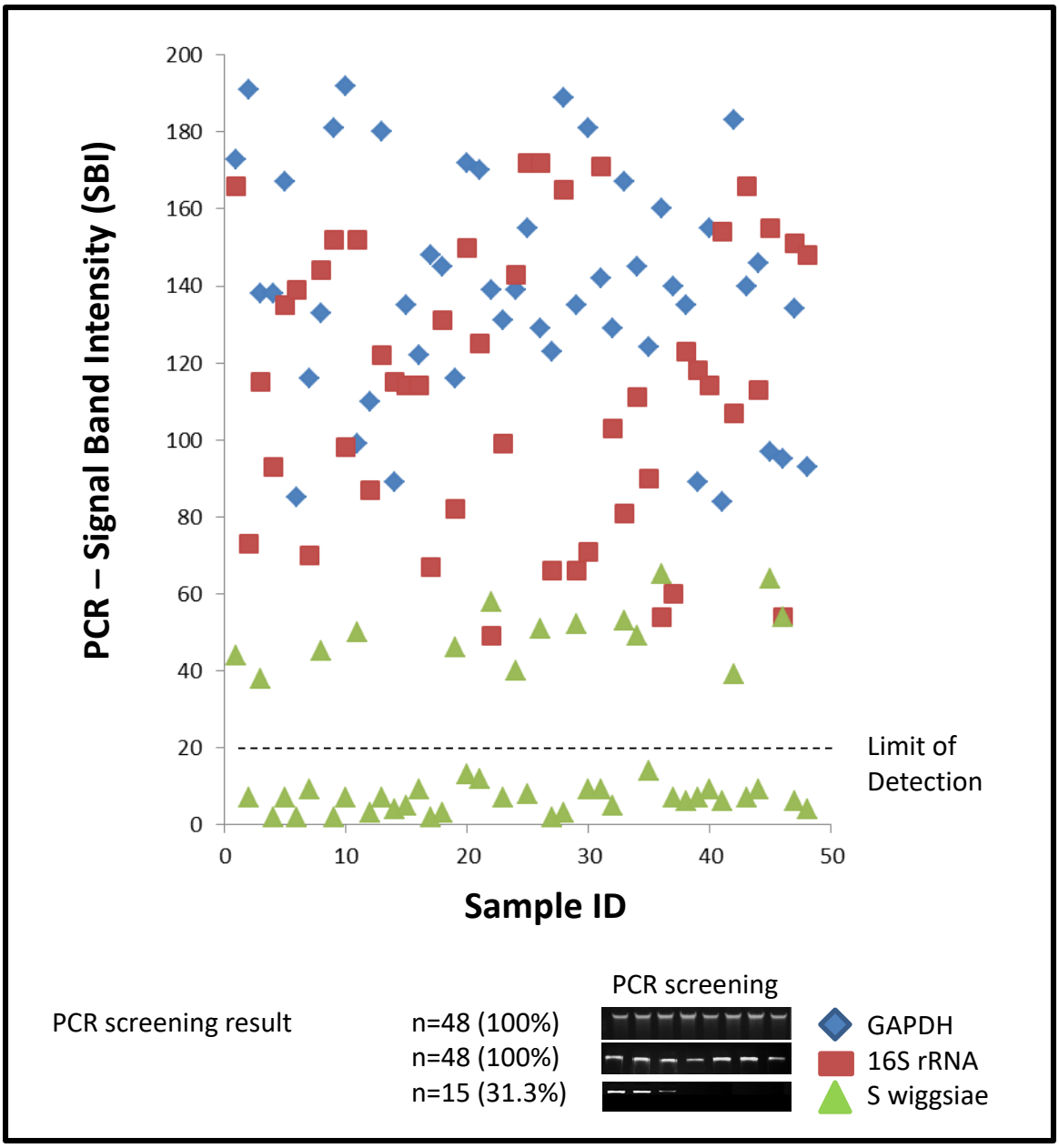

Figure 1. PCR screening for *S. wiggsiae*. DNA from each isolate was screened for human (GAPDH) and bacterial (16S rRNA) revealing positive results for all samples (n=48). PCR results for *S. wiggsiae* revealed a subset (n=15/48 or 31.25%) harbored DNA from this organism.

A more detailed analysis of the S. wiggsiae (SW)-positive and SW–negative samples was performed to determine if sex or race/ethnicity were associated with a positive screening result (Table 3). The percentage of SW-positive and SW-negative samples that were female (53.3% and 51.5%, respectively) were comparable and not significantly different (p=0.2547). In addition, the percentage of SW-positive and SW-negative samples that were derived from minority patients (60% and 57.6%) were also similar and not significantly different (p=0.1246).

Table 5. Analysis of Scardovia-positive and –negative samples.

	SW-positive (n=15)	**SW-negative (n=33)**	**Statistical analysis**
Sex			$\chi 2$=1.297
Female	53.3% (n=8)	51.5% (n=17)	d.f.=1
Male	46.6% (n=7)	48.5% (n=16)	p=0.2547
Race/Ethnicity			$\chi 2$=2.358
White	40% (n=6)	42.4% (n=14)	d.f.=1
Minority	60% (n=9)	57.6% (n=19)	p=0.1246

Discussion

Due to the increased caries risk associated with orthodontic treatment in general, and in pediatric patients more specifically, the goal of this study was to use an existing saliva repository to

identify any pediatric orthodontic patient samples that could be screened for *Scardovia wiggisae*. The results of this retrospective pilot study have revealed that a significant subset of these patients (approximately one-third) harbor DNA from this organism. These results are important as the other screening of non-Orthodontic samples from this patient population revealed a prevalence of 26.3% among pediatric patients and only 24.7% among adult patients [16]. The only screening of orthodontic patients from this patient pool was performed only among adult patients, revealing *Scardovia* among 14% of those adult Orthodontic patients compared with 19% among an age-matched sample of non-Orthodontic adult controls [18].

Although preliminary in nature, the results of this pilot study may suggest a higher percentage of pediatric orthodontic patients harbor oral *S. wiggsiae*, which may be a significant concern due to the cariogenic potential of this organism. As more studies evaluate the prevalence of Scardovia among adolescent and pediatric patient populations, more research will be needed to determine if oral alterations (such as orthodontic brackets) are capable of altering the growth and viability of these organisms [21,22]. These data will be critically important for dental clinicians and orthodontists to more accurately assess the oral health and disease potential among their patients seeking orthodontic treatment and therapy.

Although these data provide novel data regarding this patient population, this study had many limitations that must also be considered. For example, the retrospective nature of this study significantly limited the size of the potential patient pool that could be evaluated and screened. In addition, these samples were collected as part of a convenience sample that was based exclusively within a public dental school setting that focuses primarily on low income and minority patient populations [18-20]. Based upon this information, it is possible that the results

of this initial pilot study may be biased due to the nature of this patient population – although more studies will be needed to determine if these factors may be relevant.

Conclusions

As previous studies from this laboratory using adult orthodontic patients and pediatric non-orthodontic patients revealed lower prevalence – the findings of this current pilot study suggest that the newly identified cariogenic pathogen *S. wiggsiae* may disproportionately affect pediatric orthodontic patients for reasons that are not well understood. As previous research has demonstrated that oral health status and caries risk may be related to education, income, and socioeconomic status, these findings help to elucidate and contextualize the risks facing these populations – although more research will be needed to fully understand these results.

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Conflicts of Interest

The authors declare there are no conflicts of interest to report.

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Chapter 3

Prevalence of Scardovia wiggsiae among a pediatric Orthodontic patient population.

This chapter has been submitted for review and publication in the journal *EC Dental Science* and is presented in the style of that journal. The complete citation will be:

Reyes N, Pollock, A, Whiteley A, Kingsley K, Howard KM (2017) Prevalence of Scardovia wiggsiae among a pediatric Orthodontic patient population. *Current Research in Dentistry*

Role of Authors:
Dr. Adam Whiteley designed the study and worked with dental students Nicole Reyes and Alexander Pollock for the real-time PCR data generation and collection. Dr. Karl Kingsley was secondary author and assisted Dr. Whiteley with data analysis.

Abstract

Orthodontic treatment has been associated with changes in oral microbial flora, particularly among pediatric populations. Many studies have focused on the alterations in the prevalence of cariogenic pathogens, such as *Streptococcus mutans*. Recent evidence has revealed a newly discovered Gram-positive cariogenic pathogen, *Scardovia wiggsiae* – although few studies exist that explore prevalence among Orthodontic patients. Based upon this information, the primary objective of this study is to determine the prevalence of *S. wiggsiae* among pediatric Orthodontic patients from an existing saliva repository.

This retrospective screening of the existing saliva sample repository revealed n=156 pediatric (<18) samples taken from the Orthodontic clinic that were not previously screened for the presence of *S. wiggsiae*. DNA isolation was performed on n=107 samples and successfully isolated from n=72 samples, yielding a recovery rate of 67.2%. Following DNA isolation, samples with sufficient quality and quantity were screened using qPCR with primers specific for *S. wiggsiae*. This analysis revealed the presence of *Scardovia* in n=32/72 or 44.4% of

successfully screened pediatric Orthodontic patient samples, which were almost evenly distributed among Males and Females.

Although few previous studies exist to evaluate the prevalence of *Scardovia*, a previous study from this group demonstrated prevalence among pediatric patients of 26% and adult patients of 19%. Past studies also revealed the prevalence of *S. wiggsiae* in adult Orthodontic patients to be 14%. The data from this current study suggest significantly higher prevalence among pediatric Orthodontic patients, which provides new information regarding the potential changes in pathogen levels among this population. Although inference from this study is limited by the retrospective nature of this study, it may be among the first to report significant differences in *S. wiggsiae* prevalence among pediatric Orthodontic patients that may improve our understanding of cariogenic pathogens and risk among this population.

Key words: *Scardovia wiggsiae*, pediatric, Orthodontic

Background and Introduction

Cariogenic bacteria are an important focus of research as almost 50% of children and most adults in the United States (US) are affected by dental caries [1,2]. Orthodontic appliances increase the risk of developing white spot lesions, an early stage of carious lesion development [3,4]. A major reason behind the increased development of pre-carious and carious lesions is due to increased difficulty in effectively removing plaque on all tooth surfaces when orthodontic appliances are present [5,6].

Fixed orthodontic appliances may also inhibit the oral environment's innate ability to cleanse itself using salivary flow and soft tissue movements, two of the major mechanisms that help clear the mouth of food [7,8]. Plaque is one of the major niches for cariogenic bacterial growth and will contribute to the formation of white spot lesions, and eventually carious lesions [9-11]. Many studies have evaluated dental plaque to identify the major cariogenic organisms, which include the *Streptococcus mutans* and *sobrinus, Lactobacillus acidophilus, Actinomyces* spp. and *Nocardia* spp. [12,13].

Recent evidence has revealed the presence of a novel cariogenic bacterium *Scardovia wiggsiae* (SW), which was originally isolated from children with severe early childhood caries (SECC) [14,15]. More recent studies have demonstrated the presence of *Scardovia* among other children without SECC, although there is not sufficient evidence to determine the overall prevalence of this oral bacterium [16-18]. In addition, only two studies to date have sought to evaluate the presence and cariogenic potential of SW among orthodontic patients [19,20].

A recent pilot study at this institution determined that pediatric orthodontic patients may have increased probability of harboring SW, compared with adult orthodontic patients or pediatric patients without orthodontic brackets [17,20,21]. Due to the paucity of evidence regarding the prevalence of SW and the increased risk of carious lesions with orthodontic treatment, the overall goal of this project was to more thoroughly investigate the prevalence of SW among pediatric orthodontic patients within the patient population of the public dental school in Nevada. Since it has been well established that the oral environment changes because of patient related factors, a firm understanding of how the oral microbiome is changed during orthodontic

treatment is imperative in developing strategies to ensure successful risk management among patients during orthodontic treatment.. More accurate assessment of the oral microbiome and prevalence of cariogenic risk would allow for more accurate determination of cariogenic bacteria and effective patient management and treatment with more predictable treatment results.

Material and Methods

Human Subjects

This study was reviewed and approved by the Office for the Protection of Research Subjects (OPRS) Institutional Review Board (IRB) on March 7, 2016 (Protocol#880427-1 "Retrospective investigation of Prevalence of *Scardovia wiggsiae* in pediatric orthodontic patients") at the University of Nevada, Las Vegas. The original protocol for the screening of saliva samples was approved on February 6, 2015 (Protocol#1502-5068M "The Prevalence of Oral Microbes in Saliva from the University of Nevada Las Vegas (UNLV) School of Dental Medicine (SDM) pediatric and adult clinical population"). Original saliva collections took place between July 2010 and July 2016.

Study Design

This retrospective study involved previously collected saliva samples derived from a convenience sample of pediatric and adult patients recruited from the UNLV-SDM clinics. As with all clinical studies, adult participants were required to provide Informed Consent prior to collection of demographic information and saliva samples. Exclusion criteria included patients (or their appointed guardian) who declined to participate. Pediatric and Orthodontic dental residents recruited UNLV pediatric subjects between the ages of 3 and 17 years after receiving

informed consent from parents or guardians for their children to participate in the study. Although children under 18 years of age are not able to give informed consent, in Nevada, children aged 7 years and older who are able to read, comprehend, and write are asked to provide "pediatric assent," which is an agreement to voluntarily participate in research. Pediatric assent from each patient was also obtained prior to collection of demographic data and saliva samples. Patients whose parents or guardians declined to let them participate were excluded, as were patients who themselves declined to participate. Also, any child who was not a patient of record at the UNLV School of Dental Medicine clinics was excluded.

Saliva Collection

In the original study protocol, 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 concurrently collected and given the matching randomly generated number for analytical purposes, but no patient-specific identifying information was subsequently available to any research team member.

DNA isolation

DNA was isolated from each saliva sample using the GenomicPrep DNA isolation kit from Amersham Biosciences (Buckinghamshire, United Kingdom) and the procedure recommended by the manufacturer, as previously described [17,20,21,22]. DNA was suspended and stored in 50 uL DNA Hydration Solution from Amersham Biosciences (Buckinghamshire, United Kingdom) at 4C. DNA purity was calculated using ratio measurements of absorbance at 260 and 280 nm (A260/A280 ratio).

Polymerase chain reaction (PCR) primers

qPCR specifications included an initial incubation at 50C for two minutes, denaturation at 95C for 10 minutes and 40 cycles at 95C for 15 seconds and 60C for one minute [23]. Positive DNA controls were derived from previously identified SW-positive samples [17,20,21]. Primers synthesize from Eurofins MWG Operon (Huntsville, AL) were used with TaqMan universal PCR master mix, with final probe concentration at 0.2 uM using 5 uL of template (sample) DNA per reaction. The 5'-end of the *Scardovia wiggisae* probe (SwP) was labeled with 6-carboxyfluorescein (FAM) and the 3'-end with tetramethyl-6-carboxyrhodamine (TAMRA). Nuclease-free, sterile water from Promega (Madison, WI) was added to increase the final reaction volume to 25 uL. Screenings were each performed in duplicate.

Forward primer-SW, GTGGACTTTATGAATAAGC (19 bp)

Reverse primer- SW, CTACCGTTAAGCAGTAAG(18 bp)

SwP[6 ~ FAM] 5'-AGCGTTGTCCGGATTTATT-3'G [TAMRA]

Statistical analysis

Information regarding the basic demographics of the study sample were analyzed as simple descriptive statistics (counts and percentages). The basic composition of the study sample was compared with the overall composition of the clinics from which the samples were drawn to determine any significant differences in demographics between the sample group and the clinic population using GraphPad (San Diego, CA) Chi Square (χ^2) analysis online software.

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% . Using a significance level of $p = 0.05$ and a power $p = 0.80$, a minimum sample size of fifty (N = 50) was calculated [24].

Results

The existing saliva sample repository was screened for samples from pediatric patients that were undergoing orthodontic treatment, which revealed a total potential study sample size of n=156 (Table 1). The analysis of the demographic information regarding these samples revealed that slightly more than half were derived from female patients (56.4%), which is not significantly different from the overall orthodontic clinic population. However, the overwhelming majority of saliva samples identified were derived from minority patients (91%), which is significantly higher than the overall percentage from the clinic population (58.6%). The average age for the patient samples identified for this study was 13.5 years, which is lower than the overall average age for all pediatric, orthodontic patients from the clinic (15.8 years) with a range between 11 and 17 years of age.

Table 1. Demographic analysis of study participants

	Study sample (n=156)	**Clinic population**	**Statistical analysis**
Sex			
Female	n=88 (56.4%)	50.9%	χ^2=1.468, d.f.=1

Male	n=68 (43.6%)	49.1%	p=0.2257
Race/Ethnicity			
White	n=14 (9.0%)	41.4%	χ^2=60.286, d.f.=1
Minority	n=142 (91.0%)	58.6%	p<0.0001
Hispanic	n=100 (64.1%)	35.9%	
Black	n=10 (6.4%)	13.1%	
Asian/Other	n=32 (20.5%)	4.2%	
Age			
Average	13.5 yrs.	Under 18 (15.8 yrs.) Over 18 (21.4 yrs.) Combined: 18.6 yrs.	
Range	11-17 yrs.	11-38 yrs.	

DNA isolation was then performed on each of the identified samples, n=156 (Table 2). Some samples identified for this study had insufficient volume remaining to perform the DNA isolation procedure (n=49), which represented 31.4% of the potential study sample. Although DNA was isolated from n=102 samples, only n=72 had sufficient DNA quantity (>0.1 ug/mL) and sufficient DNA quality (A260:A280 ratio > 1.65) for subsequent qPCR screening. This represented only 66.4% of the previously identified samples. The percentage of samples from females and males was roughly equal at each step of the screening process (sufficient volume,

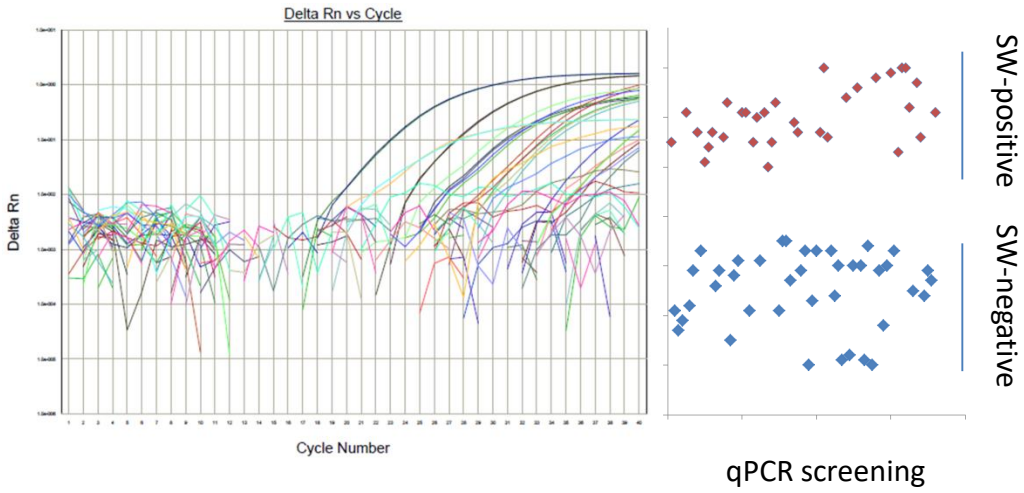
successful DNA recovery), while the percentage of samples from non-minority (White) patients remained fairly constant (~10%).

Table 2. DNA isolation and screening

	Sufficient volume	**DNA recovery**
Total samples (n=156)	n=107/156 (68.5%)	n=72/107 (67.3%)
Female	n=55/107 (51.4%)	n=39/72 (54.2%)
Male	n=52/107 (48.6%)	n=33/72 (45.8%)
White	n=11/107 (10.3%)	n=6/72 (8.3%)
Minority	n=96/107 (89.7%)	n=66/72 (91.7%)
	[DNA] =396.2 ng/uL	[DNA] =335.1 ng/uL
	A260:A280: 1.12-2.0	A260:A280: 1.65-2.0

All DNA isolates that had sufficient DNA quantity (>0.1 ug/mL) and purity (A260:A280 ratio>1.65) were then screened using qPCR for the presence of *Scardovia wiggsiae* (Figure 1). These results revealed that slightly less than half of the samples (44.4% or n=32/72) harbored DNA for this organism, with the remainder testing negative. The analysis of these data revealed

that the SW-positive and SW-negative samples were nearly equally distributed among males and females, which was similar to the overall sample composition (p=0.6877). In addition, the percentages of SW-positive and SW-negative samples that were obtained from minority patients was also similar to the overall sample composition at approximately 90% (p=0.7124).



	SW-positive (n=32)	SW-negative (n=40)	Statistical analysis
Sex			
Female	53.1% (n=17)	55.0% (n=22)	$\chi 2=0.162$, d.f.=1
Male	46.9% (n=15)	45.0% (n=18)	$p=0.6877$
Race/Ethnicity			
White	9.4% (n=3)	7.5% (n=3)	$\chi 2=0.136$ d.f.=1
Minority	90.6% (n=29)	92.5% (n=37)	$p=0.7124$

Figure 1.*Scardovia wiggsiae* qPCR saliva screening results. DNA isolates from each saliva sample were screened for of S. wiggsiae, with 44.4% (n=32/72) testing SW-positive. These were nearly equally distributed among females and males, with correspondingly similar percentages of SW-positive and SW-negative samples coming from minorities (90.6% and 92.5%, respectively).

Discussion

The primary objective of this study was to examine the oral prevalence of S. wiggsiae among pediatric patients within the patient population of the public dental school in Nevada using an existing saliva repository. Although a large number of samples were identified for inclusion in

this study (n=162), approximately one-third did not contained sufficient volume for processing, which resulted in a final sample size with sufficient DNA quality and quantity of less than half the original number (n=72). However, this was greater than the minimum sample size needed derived from the initial sample size estimated (n=50) from the power calculation.

Other studies from this institution have determined the prevalence of *S. wiggsiae* using adult and pediatric samples from this saliva sample repository and patient population. These studies demonstrated that only about one-fifth of adults and approximately one-fourth of pediatric patient saliva samples harbored DNA from this organism [17,20]. The results of this current study suggest that pediatric patients with orthodontic appliances may have increased prevalence of oral *S. wiggisae*. However, a more appropriate comparison may include an analysis SW-prevalence among other orthodontic patients.

One of these previous studies included *S. wiggisiae* screening among both adult orthodontic and non-orthodontic patients, which demonstrated prevalence of 19% and 14%, respectively. These data suggested that the prevalence among adult orthodontic patients may be lower than adults without orthodontic brackets. Although a small pilot study of pediatric orthodontic patients at this institution (n=48) revealed a somewhat higher prevalence 31.3%, the results of the current study of pediatric orthodontic patients clearly demonstrated a much higher prevalence (44.4%) of this organism than the two previous studies of non-orthodontic pediatric patients undertaken at this institution (21.3%, 26.3%), which suggests that pediatric patient populations may be at higher risk for harboring this organism while undergoing orthodontic treatment [21].

Despite the significance that this study is among the first to screen for *S. wiggiae* among pediatric orthodontic patients, there are some limitations inherent to this study design which must

also be considered. The most important of these considerations is the retrospective nature of this study, which limited the quality (and quantity) of saliva samples available for testing after long-term storage [25,26]. An additional consideration, also related to the retrospective nature of this study, is the lack of temporal information regarding *Scardovia* prevalence. For example, no longitudinal data are available to determine if the prevalence of this organism increases among the same patients after orthodontic bracket placement or if some other as yet unidentified factors may explain these results.

Based upon these factors, it is imperative that longitudinal studies of this organism be undertaken to determine if the placement of orthodontic brackets is sufficient to alter the prevalence of *S. wiggsiae* among these various groups of patients. In addition, studies that evaluate and compare these results for adults, as well as pediatric patients, are important if oral health researchers are to determine the potential for disease risks and contributions made to the oral health of orthodontic patients.

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Chapter 4

Scardovia wiggsiae prevalence among adult and pediatric orthodontic and non-orthodontic patient populations

This chapter has been submitted for review and publication in the *Journal of Medical Discovery* and is presented in the style of that journal. The complete citation will be:

A, Whiteley A, Kingsley K (2017) Scardovia wiggsiae prevalence among adult and pediatric orthodontic and non-orthodontic patient populations. *Journal of Medical Discovery*

Role of Authors:
Dr. Adam Whiteley designed the study and was the primary author, data collector and analyzer and graphics generator. Dr. Karl Kingsley was secondary author and assisted Dr. Whiteley with data analysis.

Abstract

The newly discovered cariogenic pathogen *Scardovia wiggisae* has prompted dental and oral health researchers to screen for prevalence among existing saliva repositories. Five separate studies at this institution among both pediatric and adult populations have revealed similar findings that approximately one-quarter of patients harbor this organism. The data comparing non-orthodontic patients with patients undergoing orthodontic treatment and therapy has found much higher prevalence among pediatric (but not adult) orthodontic patients. These data suggest pediatric patients may be at much higher risk although more research will be needed to contextualize and understand these results.

Key words: Scardovia wiggsiae, Pediatric, Adult Saliva Screening

Introduction

The recent discovery of a novel cariogenic pathogen *Scardovia wiggsiae* has led many scientists and oral health researchers to re-evaluate and re-examine existing saliva repositories to determine the prevalence among patient populations [1-3]. Recent efforts at this institution have used existing saliva samples to determine the prevalence among both adults and pediatric patients [4-6]. These studies have revealed this organism may be found in approximately one-quarter of all samples tested.

However, the introduction of orthodontic brackets has traditionally increased the risk of caries lesions and the growth of cariogenic organisms – which may suggest the prevalence of this organism may be different among orthodontic patient populations [7,8]. To determine if any differences could be found among the adult and pediatric orthodontic patient population, retrospective screenings of previously collected orthodontic patient saliva [9-11] were performed. These studies revealed a similar but slightly lower prevalence among adult orthodontic patients but a much higher (almost twice) prevalence among pediatric orthodontic patients [6,11,12].

Although these data have been generated from retrospective analysis of existing saliva repositories, the results to date strongly suggest an inverse, age-dependent relationship between orthodontic treatment and *Scardovia* prevalence. Higher percentages of pediatric (younger) orthodontic patients in both studies harbored this organism, while no similar finding was observed among adult orthodontic patients.

Based upon these observations a more thorough analysis and review of studies from this institution was undertaken to assess the cumulative evidence from these studies in a comprehensive and systematic manner.

Results

From the several studies undertaken at this institution, combined averages for the prevalence of *S. wiggsiae* from both pediatric and adult were plotted (Figure 1). These data clearly demonstrate that averages in oral prevalence are similar among these two populations (22% and 23%), which are similar to findings from other studies of this organism [1,7]. However, the analysis of prevalence among patients with orthodontic brackets demonstrates a significant and contrasting result. More specifically, the prevalence of adult orthodontic patients appears similar but lower than in adult or pediatric patients, while the average for pediatric orthodontic patients is nearly twice as high than non-orthodontic patients.

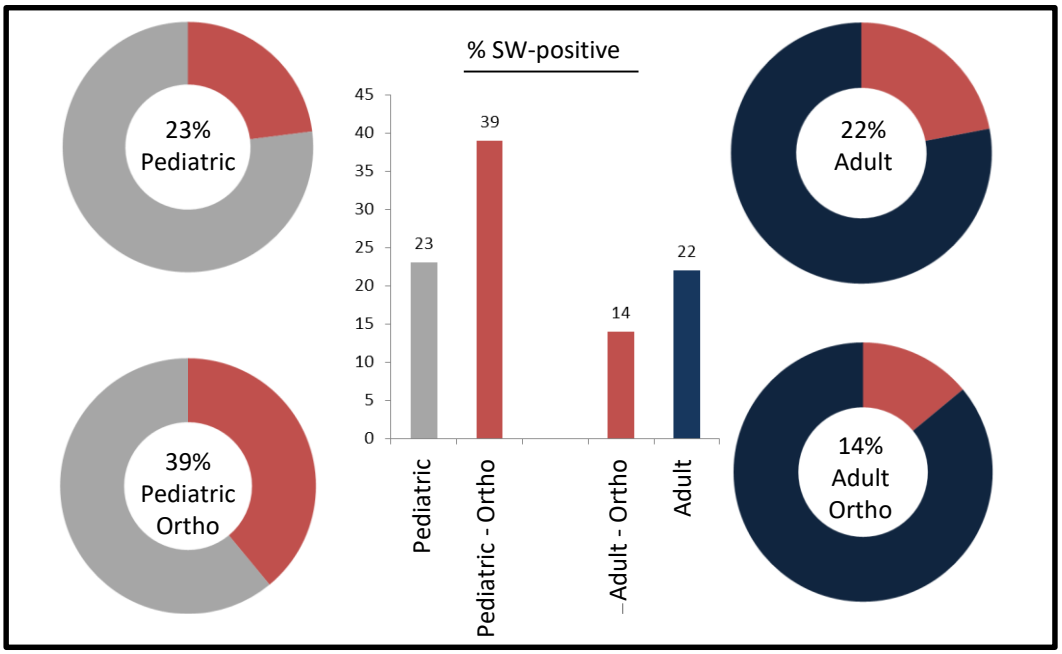

Figure 1. Analysis of combined *Scardovia wiggsiae* prevalence from UNLV-SDM studies. Data regarding *S. wiggsiae* from five studies were sorted by patient type (pediatric, adult, orthodontic, non-orthodontic) were plotted to determine average prevalence. This revealed much higher averages among pediatric, orthodontic patient saliva samples.

In order to more accurately assess the data regarding *S. wiggsiae* prevalence, specific results from each individual study were used to create a Forest plot to provide a more comprehensive analysis of this information (Figure 2). These data clearly demonstrate that although each study was completed at different times using different samples, the prevalence of *S. wiggsiae* among non-orthodontic patients was found to be within a narrow range between 19% and 26%. In contrast, the data from the two pediatric, orthodontic studies were also found to be similar but at much higher levels (between 31% and 44%).

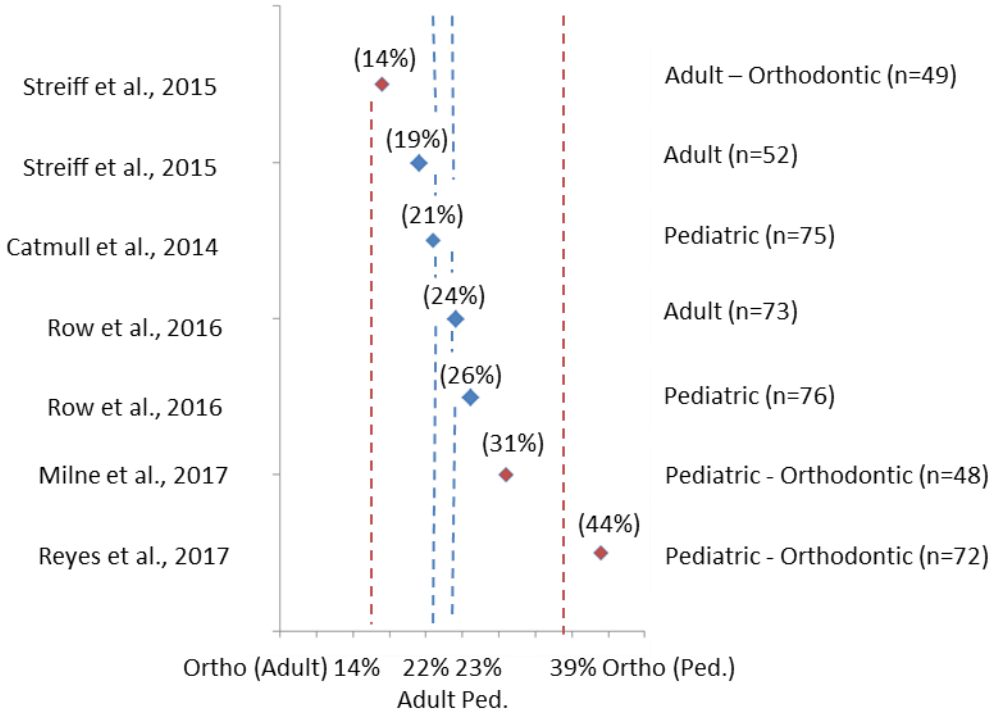

Figure 2. Forest plot of individual UNLV-SDM studies of Scardovia wiggsiae. Data for each sub-group (adult, pediatric, orthodontic, non-orthodontic) were sorted and plotted with sampe size (n) and prevalence (percentage, %). Non-orthodontic samples were found to have similar prevalence (19-26%), while orthodontic samples among pediatric patients demonstrated much higher proportions (31% and 44%).

Conclusions

Due to the recent discovery of *Scardovia wiggsiae*, few studies have gathered sufficient information to provide information regarding prevalence – particularly among high-risk populations. The combined data from each of the five studies at this institution provide strong evidence that prevalence is similar among pediatric and adult populations, however those

pediatric patients undergoing orthodontic therapy and treatment may exhibit much higher prevalence of this organism for reasons that have yet to be elucidated. More research will be needed to discovery the underlying reasons for these findings and to determine if the presence (or absence) of this organism may be related to higher or lower caries risk.

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Chapter 5: Summary and Conclusions

The purpose of this research project was two-fold – to determine the prevalence of *Scardovia wiggsiae* (SW) in pediatric patients undergoing orthodontic treatment, and to compare the prevalence SW of pediatric orthodontic patients to other populations with and without orthodontic treatment. Since SW was originally isolated from children with severe early childhood caries and is positively correlated with an increase in caries risk, it serves as an important goal to determine its prevalence in as many patient populations as possible to better assess a patient's risk for developing dental disease.

Chapter 2 of this document was a retrospective pilot study (n=48) to deliver a preliminary analysis of saliva samples obtained from pediatric patients currently undergoing orthodontic treatment. The results showed that approximately one-third of samples were positive for SW.

Chapter 3 was a larger study (n=162) that aimed to assess the prevalence of SW across all previously collected saliva samples from pediatric patients undergoing orthodontic therapy. The results from this study showed that pediatric orthodontic patients harbor SW at a much higher rate (44.4%) than originally estimated from the small pilot study in Chapter 2. Both studies are among the first to assess the prevalence of SW across different patient demographics.

Chapter 4 served to compare the results from the studies in Chapters 1 and 2 with other previously completed research on SW in other patient populations. As indicated, there is a much higher prevalence (39%) of SW in pediatric orthodontic populations when compared with adults undergoing orthodontic treatment (14%), and both adults and pediatric patients not undergoing orthodontic treatment (22% and 23% respectively).

Based on the findings presented throughout this document, in both instances the alternative hypothesis can be accepted regarding the original research questions posed at the onset of this research project.

1. Does the prevalence of *S. wiggsiae* vary between orthodontic and non-orthodontic pediatric patients?
    a. H_a: Orthodontic and non-orthodontic pediatric patients will have differences in *S. wiggsiae.*
2. Does the prevalence of *S. wiggsiae* vary between adult and pediatric orthodontic patients?
    a. H_a: Adult and pediatric orthodontic patients will have differences in *S. wiggsiae*.

Limitations and Recommendations

Being among the initial research studies to assess SW across multiple patient populations, this project lays an important foundation for continued studies. However, it is evident from all three chapters that some limitations exist, which could be improved upon in future studies to elucidate a more complete picture of SW. As this project worked with previously collected samples, it is retrospective in nature, which limits the potential patient pool that can be analyzed. Accordingly, working with samples from a previous study limited the quality and quantity of samples available for analysis. Additionally, the patient pool represented a convenience sample that was collected exclusively within a public dental school, which requires further study to determine if the results are skewed due to the nature of this patient population.

This foundation of knowledge regarding SW could be improved upon by further studies designed as longitudinal prospective studies regarding temporal information related to orthodontic treatment. These studies may include before and after delivery of fixed orthodontic

appliances, during orthodontic treatment and at intervals after debonding, and studies assessing SW prevalence with different retention protocols (bonded retention vs removable, etc). Finally, a prospective study regarding site specific SW sampling may illuminate additional information regarding the precise areas that harbor SW (gingival crevicular fluid, salivary pellicle or biofilm, adjacent to appliances, etc). While not exhaustive, there is clearly more left to be discovered about this important pathogen.

UNLV Biomedical IRB - Administrative Review
Notice of Excluded Activity

DATE:	March 18, 2016
TO:	Karl Kingsley, PhD, MPH
FROM:	UNLV Biomedical IRB
PROTOCOL TITLE:	[880427-1] Retrospective investigation of Prevalence of Scardovia Wiggsiae (SW) in pediatric orthodontic patients
SUBMISSION TYPE:	New Project
ACTION:	EXCLUDED - NOT HUMAN SUBJECTS RESEARCH
REVIEW DATE:	March 18, 2016
REVIEW TYPE:	Administrative Review

Thank you for your submission of New Project materials for this protocol. This memorandum is notification that the protocol referenced above has been reviewed as indicated in Federal regulatory statutes 45CFR46.

The UNLV Biomedical IRB has determined this protocol does not meet the definition of human subjects research under the purview of the IRB according to federal regulations. It is not in need of further review or approval by the IRB.

We will retain a copy of this correspondence with our records.

Any changes to the excluded activity may cause this protocol to require a different level of IRB review. Should any changes need to be made, please submit a Modification Form.

If you have questions, please contact the Office of Research Integrity - Human Subjects at IRB@unlv.edu or call 702-895-2794. Please include your protocol title and IRBNet ID in all correspondence.

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Appendix B

Permission to Use Copyrighted Material

University of Nevada, Las Vegas

I, **Karl Kingsley,** holder of copyrighted material entitled **Cariogenic pathogen Scardovia wiggsiae screening among pediatric orthodontic patients: A pilot study**, authored by **Weston Milne, Ghazaleh Rezaei, Adam Whiteley,** and **Karl Kingsley** originally published in **Current Research in Dentistry. August 2017** hereby give permission for the author to use the above described material in total or in part for inclusion in a Master's thesis at the University of Nevada, Las Vegas.

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I, **Karl Kingsley,** holder of copyrighted material entitled **Prevalence of Scardovia wiggsiae among a pediatric Orthodontic patient population**, authored by **Nicole Reyes, Alexander Pollock, Adam Whiteley, Katherine Howard,** and **Karl Kingsley** originally published in **EC Dental Science. August 2017** hereby give permission for the author to use the above described material in total or in part for inclusion in a Master's thesis at the University of Nevada, Las Vegas.

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Appendix D

Permission to Use Copyrighted Material

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I, **Karl Kingsley,** holder of copyrighted material entitled **Scardovia wiggsiae prevalence among adult and pediatric orthodontic and non-orthodontic patient populations**, authored by **Adam Whiteley,** and **Karl Kingsley** originally published in **Journal of Medical Discovery. September 2017** hereby give permission for the author to use the above described material in total or in part for inclusion in a Master's thesis at the University of Nevada, Las Vegas.

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September 19, 2017

Signature Date

Karl Kingsley, PhD, MPH Professor

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Chapter 1:

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Screening for the novel cariogenic pathogen Scardovia wiggsiae among Orthodontic patients

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Committee Member, Katherine Howard, Ph.D.
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