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Prevalence of Cariogenic Microbial Flora among *Scardovia wiggisiae*-Positive and Negative Patients

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PREVALENCE OF CARIOGENIC MICROBIAL FLORA AMONG *SCARDOVIA WIGGSIAE*-
POSITIVE AND NEGATIVE PATIENTS

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A thesis submitted in partial fulfillment

of the requirements for the

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Thesis Approval

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Abstract

Prevalence of Cariogenic Microbial Flora Among *Scardovia Wiggsiae*-Positive and Negative Patients

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The formation of dental caries (cavities) is a complex, multi-dimensional process that necessarily involves many risk factors – including the acquisition and colonization of cariogenic oral bacteria. The most frequently associated oral pathogens are the acid-producing and acid-tolerant oral streptococcus species, such as *Streptococcus mutans* (*S. mutans* or SM). Many studies have established and confirmed the critical role of the formation of biofilm in the virulence of *S. mutans*, and the critical role this may play in determining the balance of the oral microbiome towards health or disease.

More recent efforts have discovered a novel cariogenic pathogen, *Scardovia wiggsiae* (*S. wiggsiae* or SW) among the oral bacteria of children with severe early childhood caries. This pathogen has also been confirmed among the oral microbiota among patients with increased caries risk, such as orthodontic patients. Despite these efforts, much remains unknown about the

prevalence of this organism and the potential interactions with other cariogenic bacteria that might influence oral health or disease.

Studies from this group have surveyed the prevalence of oral microbial pathogens, such as *S. mutans* among pediatric and orthodontic populations. In addition, pilot studies to evaluate the presence of *S. wiggsiae* among this patient population have also emerged. However, to date few (if any) of these studies have performed simultaneous screenings of other organisms, such as *S. mutans* and *S. wiggsiae* to determine if the presence of either organism might be associated with differences in the prevalence of the other. The primary goal of this study was to determine the types of oral microbial associations that may exist among *S. wiggsiae*-positive and -negative patient samples.

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Chapter 1

Introduction

Background and Significance

About half of the general population is affected by dental caries [1]. High levels of *Streptococcus mutans* (SM) associated with dental decay appear to be the causative agent of caries [2]. Dental caries develop when early colonizers from the *Streptococcus* and *Actinomyces* species adhere to the tooth pellicle. Late colonizers including *Selenomonas noxia* (SN), *Tannerella forsythia* (TF), *Fusobacterium nucleatum* (FN), and *Treponema denticola* (TD) contribute to the progression of dental caries [3]. Recent evidence has identified another cariogenic pathogen, *Scardovia wiggsiae* (SW), present in the oral flora of a smaller subset of dental patients [4]. Although studies have been conducted to study the prevalence of SW in both pediatric and adult populations, the prevalence of cariogenic microbial flora among SW-positive and -negative orthodontic patients have not been studied. The aim of this study was to assess the prevalence of oral microbial flora including SN, SM, TF, FN, AA in SW-positive and -negative patients within a dental school population. To better study the oral microbial associations that exist between SW, this paper is divided into chapters that will help give further insight on these relationships. Chapters 2, 3, and 4 are individual studies that have been published in separate journals that help elucidate such relationships.

Selenomonas noxia and *Streptococcus mutans* remain two of the most prevalent cariogenic pathogens to date. They are associated with poor oral health and oral prevalence of these organisms may be useful as biomarkers to determine patient oral health. Current studies reveal

novel insights into the epidemiology of *S. wiggsiae*, although few studies have explored the oral microbial ecology with respect to this oral pathogen. Based upon the lack of information regarding the oral microbial ecology, this study screened an existing saliva repository to more accurately assess the microbial flora present (or absent) including *S. wiggsiae* and more specifically *S. noxia* and *S. mutans*. The findings from this study are published in the Microbiology Research Journal International and the Journal of Advances in Microbiology and presented in Chapters 2 and 3 of this paper respectively.

The prevalence of *S. wiggsiae* in the context of additional caries risk factors, such as orthodontic treatment has yet to be fully explored. Fixed orthodontic appliances influence the quantity and quality of cariogenic microbial flora. Fixed appliances may prevent the oral cavity's innate ability to self cleanse through salivary flow and soft tissue movements of the cheeks, lips, and tongue. Standard oral hygiene practices may not be sufficient to remove plaque accumulation on teeth especially around bracket interfaces. Specific changes in the oral environment results in increased plaque accumulation, microbial colonization, and development of pre-carious and carious lesions [5]. About 73% of orthodontic patients develop at least one new lesion during the duration of their orthodontic treatment [6,7]. Thus, it is important to understand how dental caries form and which causal bacteria are involved in the process so that clinicians are better able to assess caries risk factors and find preventative techniques to reduce the disease process. Recent studies at this institution have characterized the microbial ecology of *Scardovia wiggsiae*-positive and -negative saliva samples within a dental school setting. The findings from this study are published in the Journal of Scientific Discovery and presented in Chapter 4 of this paper.

Materials and Methods

A retrospective analysis of previously collected saliva samples from pediatric and adult patients will be used. Samples from the previous study (Protocol OPRS #1502-5068M: The Prevalence of Oral Microbes in Saliva from the University of Nevada, Las Vegas School of Dental Medicine (SDM) pediatric and adult clinical population) approved on February 6, 2105 will be used.

In brief, patients from the pediatric, orthodontic, and general UNLV-SDM clinics were asked to participate in the study. Patients (and parents or guardians) who participated in the study were required to provide informed consent, while pediatric patients were required to provide pediatric assent (written consent to participate). Exclusion criteria included any parent, guardian or patient (pediatric or adult) who declined to participate. Subjects who agreed to participate were given a small, sterile saliva collection container, 50mL sterile polypropylene tube (Fisher Scientific: Fair Lawn, New Jersey, USA) and asked to spit into it for a 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, DNA was isolated from these samples and subsequently screened for *Scardovia wiggisiae* (SW), *Streptococcus mutans* (SM), *Selenomonas noxia* (SN), *Tannerella forsythia* (TF), *Fusobacterium nucleatum* (FN) using real time polymerase chain reaction (qPCR) and primers specifically designed to distinguish these organisms.

Research Questions

1. Does the prevalence of cariogenic microbial flora vary in SW-positive patients?

H₀: The prevalence of cariogenic microbial flora in SW-positive patients is similar.

H_A: The prevalence of cariogenic microbial flora in SW-positive patients is different.

2. Does the prevalence of SM vary in SW-positive and negative patients?

H₀: The prevalence of SM in SW-positive and negative patients is similar.

H_A: The prevalence of SM in SW-positive and negative patients is different.

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.

Statistical Analysis

Because the difference in prevalence between groups (SW-positive, SW-negative) 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.

The analyses involving multiple two sample t-tests have a higher probability of Type I error, leading to false rejection of the null hypothesis, H_0 . To confirm the effects observed from these experiments and minimize the possibility of Type I error, further analysis of the data will be facilitated using ANOVA with SPSS (Chicago, IL) to more accurately assess relationships and statistical significance among and between groups.

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Chapter 2

Oral Microbial Ecology Of *Selenomonas Noxia* And *Scardovia Wiggsiae*

Introduction

Selenomonas noxia is an organism within the *Veillonella* family, which is widely distributed among various animal species [1-3]. Although *S. noxia* is commonly found in the gastrointestinal tract, it is also found in the oral cavity and may be found in higher levels among patients with poor oral health [4-6]. The recent developments of a rapid PCR-based detection assay and anaerobic culturing conditions have made the screening for oral *S. noxia* more accessible and cost effective [7-9].

Many patients with poor oral health often harbor multiple disease-causing organisms that may contribute to one or more pathologies within the oral cavity [10-12]. Recently, a new oral pathogen *Scardovia wiggsiae* was discovered in patients with poor oral health [13,14]. Although some screenings for *Scardovia* are now beginning to emerge, much remains to be discovered about the oral ecology and microbial interactions that facilitate or inhibit the growth of this organism [15-17].

Based upon the lack of information regarding the oral microbial ecology of these specific organisms, the primary objective of this study was to screen an existing saliva repository to more accurately assess the microbial flora present (or absent) with *S. wiggsiae*, with specific emphasis on *S. noxia*.

Methodology

Project Approval

Approval for this study was granted by the Institutional Review Board (IRB) and the Office for the Protection of Human Subjects (OPRS) under Protocol #875879-1 “*Selenomonas noxia*

prevalence in DNA previously isolated from pediatric patient saliva samples” in March 2016. The retrospective nature of this study qualified as “exempt” according to federal regulatory statute 45CFR46.101(b).

The saliva repository was originally created under the OPRS-IRB Protocol #1305-4466M “The Prevalence of Oral Microbes in Saliva from the UNLV School of Dental Medicine Pediatric and Adult Clinical Population” approved in June 2013. Briefly, this involved the collection of unstimulated saliva from pediatric and adult patients from the University of Nevada, Las Vegas (UNLV) School of Dental Medicine (SDM) dental clinic. Patients (and parents or guardians) were required to provide informed consent, while pediatric patients were also required to provide pediatric assent (written consent to participate). Exclusion criteria included any parent, guardian or patient (pediatric or adult) who declined to participate.

Saliva Repository

During the original saliva collection, demographic information (age, sex, and race/ethnicity) and saliva samples were obtained from approximately 250 patients. In brief, each study participant was provided a sterile 50 mL saliva collection container with a target of collecting 5mL. All samples were placed on ice until transfer and storage in the biomedical research laboratory. Each sample was given a unique, randomly generated unique identifier to prevent any personal or patient information from accompanying the sample outside of the clinic. No patient-specific identifying information was subsequently available to any member of this research project.

DNA Isolation

The current study involved a retrospective analysis of saliva samples available in the repository. All the available (remaining) samples (n=240) were located and then evaluated to ascertain if enough saliva remained for the DNA isolation required to perform the PCR screening. Out of the 240 samples identified, a smaller subset of n=162 contained sufficient volume (>0.5 mL) for inclusion in this study. Isolation of DNA was facilitated using the Amersham Bioscience

(Buckinghamshire, UK) GenomicPrep DNA isolation kit. The DNA from each sample was suspended in 50 uL of DNA hydration solution from Amersham Bioscience (Buckinghamshire, UK) and stored at 4C. Quality and quantity of the DNA isolated from each sample was calculated using measurements of absorbance at 260 and 280 nm to calculate the A260:A280 ratio.

PCR Screening

All DNA isolates with sufficient quantity (1 ng/uL or greater) and sufficient quality (A260:A280 ratio >1.65) were screened using qPCR to assess the presence of several oral microbial species. The qPCR used an initial incubation of 50C for 2 minutes, followed by 10 minute denaturation at 95C and 40 cycles at 95C for 15 seconds and 60C for 1 minute. The DNA positive controls were *S. noxia* reference strains obtained from American Type Culture Collection (ATCC)-43541,-51893,-700225), as previously described [7]. The positive DNA controls for *S. wiggisiae* were derived from previously identified SW-positive samples, as previously described [15,18,19]. TaqMan universal PCR master mix with the following primers from Eurofins MWG Operon (Huntsville, AL) was used, resulting in a final probe concentration of 0.2 uM with 5 uL of template (sample) DNA in each reaction. Sterile, nuclease-free distilled water from Promega (Madison, WI) was used to adjust the final reaction volume to 25 uL. Each screening was performed in duplicate.

S. noxia Forward primer- SNF1, TCTGGGCTACACACGTACTACAATG (25 bp)

S. noxia Reverse primer- SNR1, GCCTGCAATCCGAACTGAGA (20 bp)

SnP[6 ~ FAM]CAGAGGGCAGCGAGAGAGTGATCTTAAGC [TAMRA]

S. wiggisiae Forward primer-SW, GTGGACTTTATGAATAAGC (19 bp)

S. wiggisiae Reverse primer- SW, CTACCGTTAAGCAGTAAG (18 bp)

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

The selected probes (SnP, SwP) were labeled with the reporter dye 6-carboxyfluorescein (FAM) at the 5'-end and with the reporter dye tetramethyl-6-carboxyrhodamine (TAMRA) at the 3'-end.

Statistical Analysis

The information regarding this retrospective sample were summarized using simple descriptive statistics and analyzed using Chi Square (χ^2) analysis software from GraphPad (San Diego, CA) [20].

Results

All potential samples (n=162) were identified and their demographic information was compiled for analysis (Table 1). In brief, the study sample was comprised of nearly equal numbers of males and females, which is similar to the patient composition from the clinic population ($p=0.5484$). The demographic analysis by race or ethnicity, however, revealed the overwhelming majority of samples were derived from minority patients (mostly Hispanic), which is significantly higher than the proportion of minorities from the clinic population ($p<0.001$). The ages of patients included in the study sample ranged from 5 – 73 years of age, compared with 2 – 91 years of age from the general clinic population.

Table 1. Study Sample Demographic Information

	Sample (n=162)	UNLV-SDM Clinic	Analysis
Sex			
Female	n=87 (53.7%)	50.9%	$\chi^2=0.360$ d.f.=1
Male	n=75 (46.3%)	49.1%	$p=0.5484$
Race/Ethnicity			
White	n=26 (16.0%)	41.4%	$\chi^2=25.837$ d.f.=1
Minority	n=136 (83.9%)	58.6%	$p<0.001$
Hispanic	n=91 (56.2%)	35.9%	
Black	n=24 (14.8%)	13.1%	
Asian/Other	n=21 (13.0%)	4.2%	
Age Range			
	5 – 73 yrs.	2 – 91 yrs.	

DNA from each of the study samples was extracted, which revealed many samples had either insufficient quantity or insufficient quality for further processing (Table 2). More specifically, out of the total number of potential samples identified, only n=48/162 or 29.6% had sufficient DNA quality with an absorbance ratio (A260:A280) ratio within the manufacturer range 1.70 – 2.00 and sufficient DNA quantity (100-1000 ng/uL) for further processing.

Table 2. Analysis of DNA from Study Sample

	DNA Recovery	DNA Quantity	DNA Quality
Study Samples	n=48/162 (29.6%)	631.2 ng/uL +/-51.3	A260:A280 ave=1.72
Manufacturer	90-95%	100-1000 ng/uL	A260:A280 1.70-2.00

Each of the samples containing DNA of sufficient quantity and quality was processed using qPCR (Figure 1). The analysis of this screening revealed that only a small proportion of the overall study sample harbored DNA specific for *S. noxia* (n=6/42 or 14.3%). This data was

significantly different for DNA specific to *S. wiggsiae*, which was found in a much higher number of patient samples (n=27/42 or 64.3%). These results were verified by subsequent screening in duplicate.

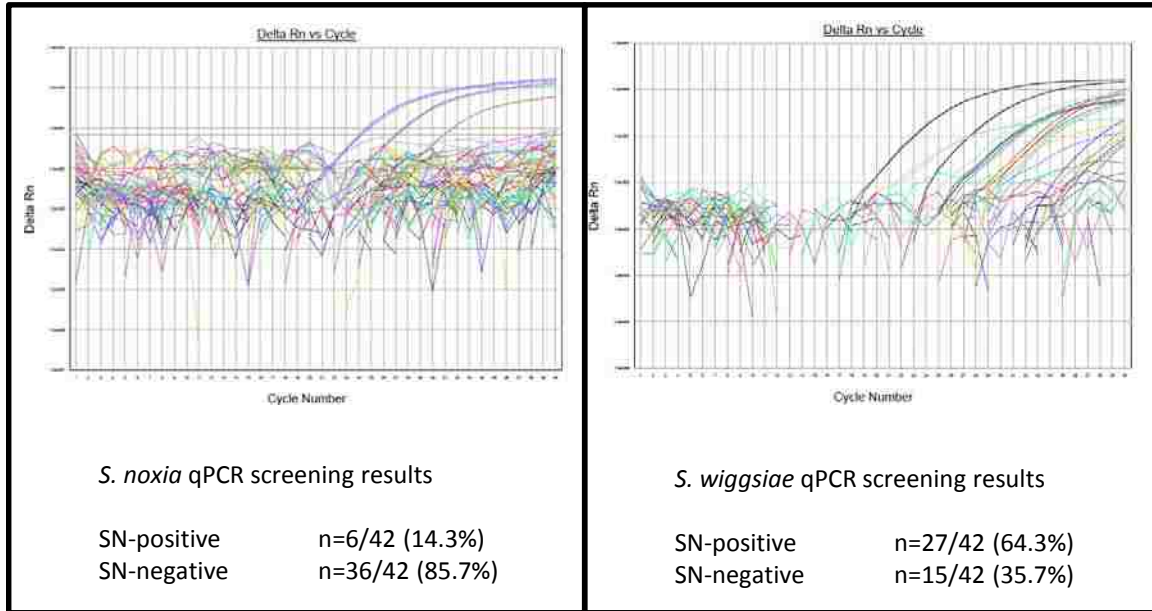


Figure 1. qPCR Screening for *Selenomonas* and *Scardovia*. qPCR screening of patient samples revealed differing prevalence of these organisms, with 14.3% of patient samples testing positive for DNA from *S. noxia*, while 64.3% tested positive for *S. wiggsiae*.

A more detailed analysis revealed that none of the samples that tested positive for *S. wiggsiae* (n=27/42 or 64%) harbored DNA for *S. noxia* (Figure 2). Conversely, the samples that tested positive for *S. noxia* also tested negative for *S. wiggsiae* (n=15/42 or 35%). In addition, although *A. actinomycetemcomitans* was only present in a small subset of samples, this organism was only found among SW-positive samples. Other organisms, including *T. forsythia* (TF), and *F.nucleatum* (FN) were present in both SW-positive and SW-negative samples although their prevalence differed greatly.

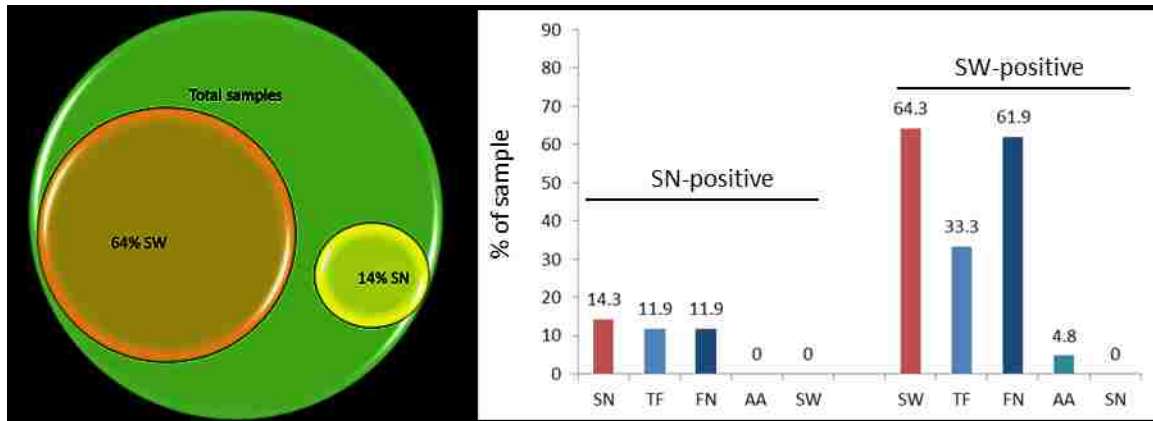


Figure 2. Analysis of SW- and SN-Positive Samples. Detailed analysis revealed that SW-positive samples (64%) did not harbor SN, while SN-positive samples (14%) did not harbor SW. Differential results were obtained from screening for other organisms, including *T. forsythia* (TF), *F. nucleatum* (FN) and *A. actinomycetemcomitans*.

Conclusion

This study may be the first to present oral microbial data which suggest SW may participate in direct or indirect bacterial interactions that influence the potential for other organisms to flourish within the oral microbiome. This data may suggest that SN and SW may occupy distinct, non-overlapping niches, which may differ significantly from the interactions observed with *A. actinomycetemcomitans*, *F. nucleatum*, and *T. forsythia*. Further research will be needed to fully elucidate these interactions and to explore the potential ramifications for oral microbial ecology and the implications for predictive saliva screening.

Discussion

The primary objective of this study was to screen an existing saliva repository to more accurately assess the microbial flora present (or absent) with *S. wiggisiae*, with specific emphasis on *S. noxia*. This screening revealed that more than half of the samples evaluated harbored *S. wiggisiae*, with a much smaller subset of samples testing positive for *S. noxia*. Interestingly, none

of the *Scardovia*-positive samples had detectable levels of DNA for *S. noxia*, while none of the *Selenomonas*-positive samples tested positive for *S. wiggsiae*.

These results may suggest that some oral microbial communities may facilitate the growth of specific species, while inhibiting the growth of others [21,22]. The observation that *A. actinomycetemcomitans* was found only among the *Scardovia*-positive samples, while differential results were observed for *F. nucleatum*, and *T. forsythia* only strengthens the evidence for this hypothesis [23,24]. While more evidence will be needed to validate these findings, this study may be the first to present oral microbial data which suggest that either *S. wiggsiae* or *S. noxia* (or possibly both) may participate in direct or indirect bacterial interactions that influence the potential for other organisms to flourish within the oral microbiome.

Due to the retrospective nature of this study, some limitations were inherent and should also be considered. For example, the length of storage time for these saliva samples may have varied greatly, which has been demonstrated to significantly affect both the quality and quantity of DNA isolates [25,26]. In addition, the higher proportion of minority patient samples from this public dental school patient population may also have influenced these results – mainly due to the limited resources and low socioeconomic status of the majority of these clinic patients [27-29].

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Competing Interests

The authors have declared that no competing interests exist.

Author Contributions

SM and JM were responsible for sample identification, DNA isolation, and experimental protocol. AT, KK and KM were responsible for project design, funding, data analysis, and manuscript preparation.

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Chapter 3

Screening a Saliva Repository for *Scardovia wiggsiae* and *Streptococcus mutans*: A Pilot Study

Introduction

The formation of dental caries (cavities) is a complex, multi-dimensional process that necessarily involves many risk factors – including the acquisition and colonization of cariogenic oral bacteria [1,2]. The most frequently associated oral pathogens are the acid-producing and acid-tolerant oral streptococcus species, such as *Streptococcus mutans* (*S. mutans*) [3,4]. Many studies have established and confirmed the critical role of the formation of biofilm in the virulence of *S. mutans*, and the critical role this may play in determining the balance of the oral microbiome towards health or disease [5-7].

More recent efforts have discovered a novel cariogenic pathogen, *Scardovia wiggsiae* (*S. wiggsiae*) among the oral bacteria of children with severe early childhood caries [8-10]. This pathogen has also been confirmed among the oral microbiota among patients with increased caries risk, such as orthodontic patients [11,12]. Despite these efforts, much remains unknown about the prevalence of this organism and the potential interactions with other cariogenic bacteria that might influence oral health or disease [13-15].

Studies from this group have surveyed the prevalence of oral microbial pathogens, such as *S. mutans* among pediatric and orthodontic populations [16,17]. In addition, pilot studies to evaluate the presence of *S. wiggsiae* among this patient population have also emerged [12,18,19]. However, to date few (if any) of these studies have performed simultaneous screenings of both *S. mutans* and *S. wiggsiae* to determine if the presence of either organism might be associated with differences in the prevalence of the other. The primary goal of this study was to determine if this type of association may exist among the oral microbiota of patient samples obtained from an existing saliva repository.

Methodology

Study Design

This was a retrospective study to evaluate the presence of both *S. wiggisiae* and *S. mutans* among patient samples from an existing saliva repository using qPCR. SW-positive (n=27) and SW-negative (n=15) samples were subsequently screened for the presence of SM. The samples were nearly evenly divided between males and females (45%, 55%, respectively) and were mostly Hispanic minorities (n=22/42 or 52%). Approval for this study was granted from the Institutional Review Board (IRB) under 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. Samples were originally collected between 2010 and 2016. In brief, each sample was given a unique, non-duplicated randomly generated identification number to protect patient anonymity – with only basic demographic information (age, sex, race/ethnicity) noted at the time of saliva collection.

Sample Selection

Patient saliva samples that were previously identified as harboring *S. wiggisiae* DNA were selected for screening using qPCR (n=27). Selected samples that were previously identified as not harboring *S. wiggisiae* DNA were also identified from patient collections during the same time period (n=15), which would reduce the potential for time-dependent degradation of samples to influence the outcome and final results. DNA was then isolated from each clinical sample using the Amersham Biosciences GenomicPrep DNA isolation kit and the manufacturer recommended protocol, as previously described [20-22].

qPCR Screening

DNA screening was accomplished using primers specific for each organism [17,23]. The probes for *S. wiggisiae* (SwP) and *S. mutans* (SmP) were each labeled with 6-carboxyfluorescein (FAM) at the 5' end and with tetramethyl-6-carboxyrhodamine (TAMRA) on the 3-end, as specified:

Forward primer-SW, GTGGACTTTATGAATAAGC (19 bp)

Reverse primer-SW, CTACCGTTAAGCAGTAAG(18 bp)

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

Forward primer-SM, GCCTACAGC TCAGAGATGCTATTCT (26 bp)

Reverse primer-SM, GCCATACACCACTCATGAATTGA (23 bp)

SmP [6 ~ FAM] 5'-GAAACCAACCCAACTTTAGCTTGGAT-3'G [TAMRA]

All qPCR reactions were performed using TaqMan universal PCR master mix with the probe concentration at 0.2 uM and a minimum of 5.0 uL of target (sample) DNA. All reactions were performed in duplicate using incubation at 50C (2 min), denaturation at 95 C (10 min), 40 cycles at 95C (15 sec) and 60C (1 min).

Statistics

Demographic information was summarized and presented as simple, descriptive statistics (both number and percentage). The composition of the study sample was compared with the overall clinic composition from which it was originally collected and these data were analyzed using Chi Square (χ^2) software from GraphPad (San Diego, CA) [24]. This analysis was also used to analyze the qPCR results.

Results

The demographic analysis revealed that the study samples (n=42) were derived from nearly equal numbers of females and males (Table 1). However, the racial and ethnic composition of the study sample had a much higher proportion from minority patients than the overall clinic population from which that sample was collected ($p=0.0005$). The study sample was comprised of both pediatric and adults, ranging in age from 12 – 41 years.

Table 1. Study Sample

	Study Sample (n=42)	Clinic Population	Statistical Analysis
Sex			
Male	45.2% (n=19)	49.1%	$\chi^2=0.640$, d.f.=1 $p=0.4236$
Female	54.8% (n=23)	50.9%	
Ethnicity / Race			
White	23.8% (n=10)	41.4%	$\chi^2=11.947$, d.f.=1 $p=0.0005$
Minority	76.2% (n=32)	58.6%	
Hispanic	52.4% (n=22)	35.9%	
African American	16.7% (n=7)	13.1%	
Other	7.1% (n=3)	4.2%	
Age Range			
	12-41 yrs.	2 – 91 yrs.	

DNA was subsequently isolated from the pre-selected samples, which was within the range specified by the manufacturer (Table 2). Successful isolation was accomplished and the quality assessed using absorbance readings at 260 and 280 nm (A260:A280 nm ratio). Quantity of DNA was also determined to be sufficient for qPCR screening of all samples.

Table 2. DNA Isolation and Analysis

	Study Sample (n=42)
DNA Recovery	n=42/42 (100%) Range (manufacturer estimate) 95-100%
DNA Purity	A260:A280 range: 1.51 – 2.00 Ave: 1.71 Acceptable range (manufacturer) 1.65 – 2.00
DNA Concentration	[316.2 ng/uL] range: 91.2 – 873.4 Manufacturer range: 100 – 1000 ng/uL

The screening of study samples using qPCR was performed, which revealed differential results for each of the organisms evaluated (Figure 1). More specifically, qPCR screening of the previously identified *S. wiggisiae*-positive samples confirmed n=27 samples harbored DNA from this organism. qPCR analysis confirmed the *S. wiggisiae*-negative samples (n=15) and revealed only a small fraction (27%) harbored DNA from *S. mutans* (Fig 1B).

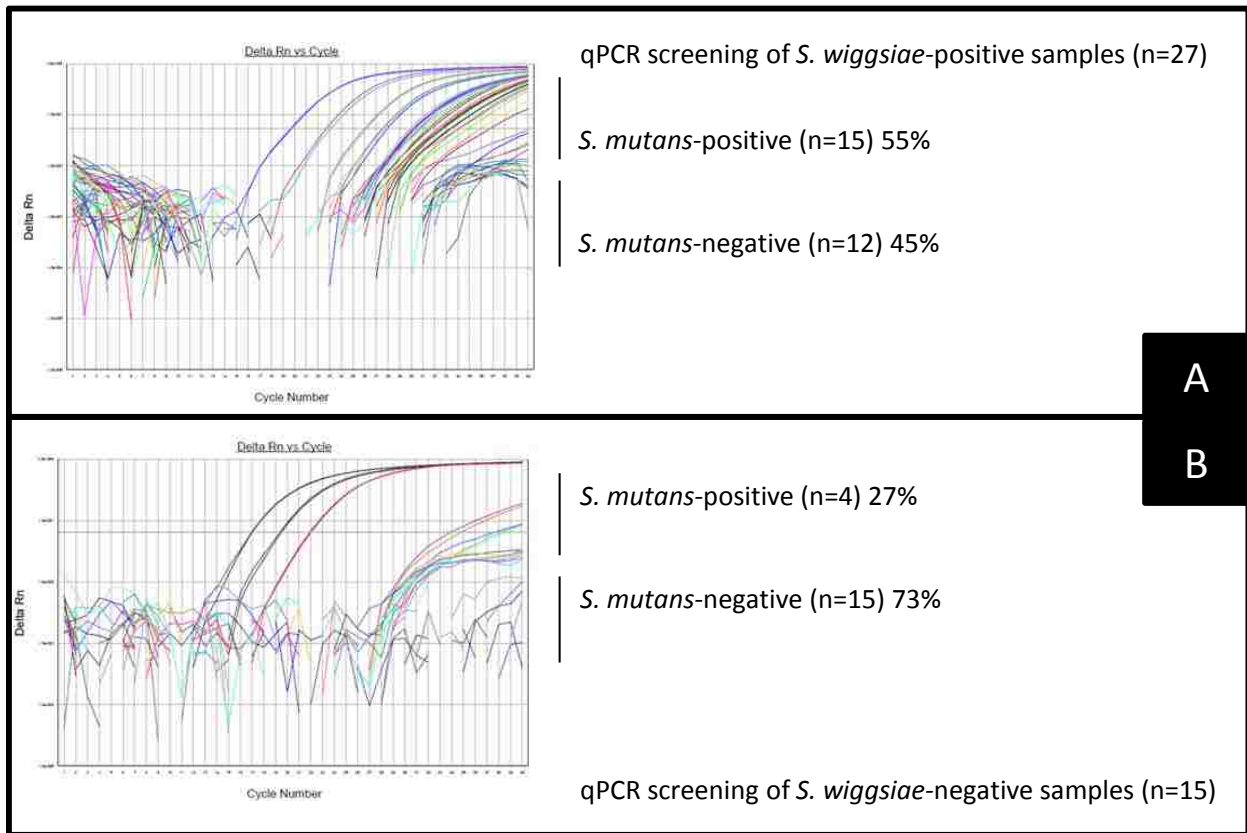


Figure 1. Screening of Study Samples Using qPCR. A) Screening of *Scardovia*-positive samples revealed more than half also harbor *S. mutans* DNA (55%). B) Screening of *Scardovia*-negative samples revealed relative few also contain DNA from *S. mutans* (27%).

A more detailed analysis of these results revealed that 45% of samples (n=19/42) harbored SM. The vast majority of *S. mutans*-positive samples (n=15/19 or 79%) were derived from *S. wiggisiae*-positive samples (Figure 2). Only a small percentage of *S. mutans* positive samples (n=4/19 or 21%) were derived from the *S. wiggisiae*-negative samples. Chi-square analysis of these results strongly suggests this distribution was unlikely due to chance ($p < 0.00001$).



Figure 2. Statistical Analysis of *S. wiggisiae* and *S. mutans* Screening. Although *S. mutans* was found in both *Scardovia*-positive and -negative samples, detailed analysis of the qPCR screening results demonstrates a statistically significant difference was observed among the *Scardovia*-negative samples, with a much lower percentage also harboring *S. mutans* ($p<0.0001$).

Conclusion

The limited numbers of studies available regarding *S. wiggisiae* prevalence have suggested that *S. wiggisiae* and *S. mutans* may inhabit similar and overlapping niches within the oral microbiome. In fact, some work has suggested the potential for competition and interactive inhibition between these organisms within the oral cavity. The preliminary data from this pilot study suggest *S. mutans* and *S. wiggisiae* may, in fact, be present in the same patients and may not therefore be exclusively competitive – at least in this cross sectional study. However, due to the large differences observed among these samples, further research will be needed to further elucidate and validate these findings.

Discussion

The primary goal of this study was to perform highly sensitive qPCR screenings of patient saliva samples for both *S. mutans* and *S. wiggsiae* to determine if the presence of either organism might be associated with differences in the prevalence of the other using an existing saliva repository. This pilot study has revealed that both cariogenic pathogens *S. mutans* and *S. wiggsiae* may, in fact, be present in the same patients and are therefore unlikely to be exclusively competitive [25,26]. However, the discrepancy in the prevalence of *S. mutans* among *S. wiggsiae*-positive and -negative samples may suggest alternative factors may be influencing the microbial composition of patient oral flora [27,28].

Recent studies have now demonstrated that the salivary microbiota differ significantly among patients with different caries risk and experience [29,30]. This evidence has provided increasing support for the hypothesis that co-association of cariogenic and pathogenic oral microbes, may be understood more clearly as moving in tandem and providing commensal opportunities rather than merely as competitors for limited resources and available space [31,32]. Although much remains to be discovered regarding the epidemiology of these organisms in oral disease and prevention, these data suggest a more thorough understanding of prevalence will help clinicians and healthcare providers in both disease prevention and treatment.

Although these findings represent novel information regarding co-association and prevalence of *Scardovia* in relationship to *S. mutans*, there are some limitations of this study that must be considered. First, the retrospective nature of this study did not allow for the collection of samples based upon caries risk or caries experience, which may represent a significant confounding variable. In addition, the original establishment of the saliva repository was done as a convenience sample at a low-income, public university-based dental school – which may represent patients with more limited access to healthcare and more likelihood to have low levels of health literacy and insurance [33-35].

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Competing Interests

The authors have declared that no competing interests exist.

Author Contributions

JM and SM were responsible for sample identification, DNA isolation, PCR data generation, and experimental protocol. AT, KK and KM were responsible for project design, funding, data analysis, and manuscript preparation.

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Chapter 4

Microbial Ecology of *Scardovia wiggsiae*-Positive and Negative Samples

Introduction

The studies from this institution were initiated to reveal whether samples from pediatric (and some adult) patients harbored DNA specific for *Scardovia wiggsiae* (*S. wiggsiae*) [1]. Although the first descriptions of this organism were from children with severe early childhood caries, the main finding from this initial pilot study was the discovery of *S. wiggsiae* in approximately one-quarter of both the pediatric and adult patient saliva samples [2,3]. A more recent study from this group confirmed these findings among a much larger sample of both pediatric and adult patients, further support for the growing evidence that *Scardovia* may be part of the oral microbial flora in patients with severe early childhood caries, as well as in pediatric and adult patients with other caries risk factors and profiles [4-6].

Some evidence has suggested *S. wiggsiae* may be a smaller part of the normal oral flora in patients without caries [7,8]. However, other studies have now demonstrated that orthodontic therapy may increase the risk of both caries and of high levels of *Scardovia* in some patients [9]. This observation has also been made in studies from this group, which has demonstrated the presence of this organism in nearly twice the percentage of pediatric orthodontic patients compared with either adult orthodontic patients or pediatric patients without orthodontic appliances [10]. In fact, two additional studies of orthodontic patients have recently been completed, which provide more support for these observations [11,12].

These studies provide the rationale for a more thorough investigation and screening of patient samples, which have been demonstrated to harbor *S. wiggsiae* [13,14]. Based upon these studies, sufficient data now exist to provide a more detailed analysis and description of the microbial ecology found among *Scardovia*-positive and -negative patients samples.

Results

Using the previous microbial screening studies from this institution, results for the prevalence of several oral microbial species were compiled for analysis (Figure 1). This data clearly demonstrate that bridge species, such as *Fusobacterium nucleatum* (FN) are present in the overwhelming majority of samples – supporting other similar observations [15-17]. This data demonstrate that both the cariogenic pathogens *S. wiggisiae* (SW) and *Streptococcus mutans* (SM) are found in nearly half of all patient samples [1,4,9,11-14]. However, other oral pathogens, such as *T. forsythia* (TF) and *Selenomonas noxia* (SN) were only found in approximately one-quarter of patient samples [13,18].

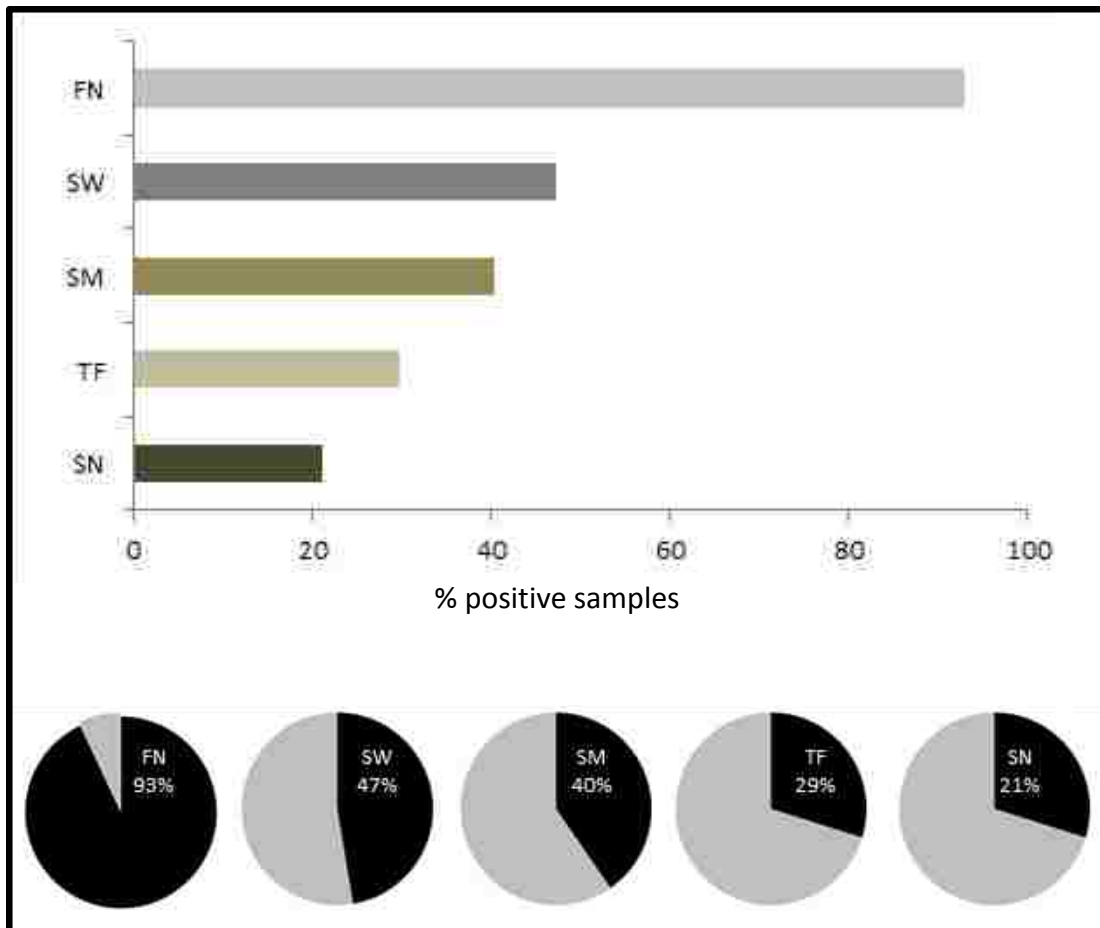


Figure 1. Combined Analysis of Institutional Screening Studies of Oral Microbial Pathogens. An analysis of all patient saliva screening studies revealed most samples harbored DNA from the

periodontal pathogen *F. nucleatum* (FN), while smaller subsets were found to contain the cariogenic pathogens *S. wiggsiae* (SW) and *S. mutans* (SM). Additional oral microbes *T. forsythia* (TF) and *S. noxia* (SN) were also present in approximately one-quarter of all samples analyzed.

While these data provide some limited information regarding prevalence for several important oral species, more detailed analysis of data specific to each sample screened for multiple studies can provide a more comprehensive profile of the microbial flora (Figure 2). More specifically, a subset of samples that were screened in multiple studies provides a detailed analysis of the major pathogenic organisms, such as *S. wiggsiae* and *S. mutans*, which were present in nearly half of the same samples – either alone or in combination with *F. nucleatum*. These data clearly demonstrate that although *S. wiggsiae* and *S. mutans* are commonly found with *F. nucleatum*, they may also both be found concomitantly.

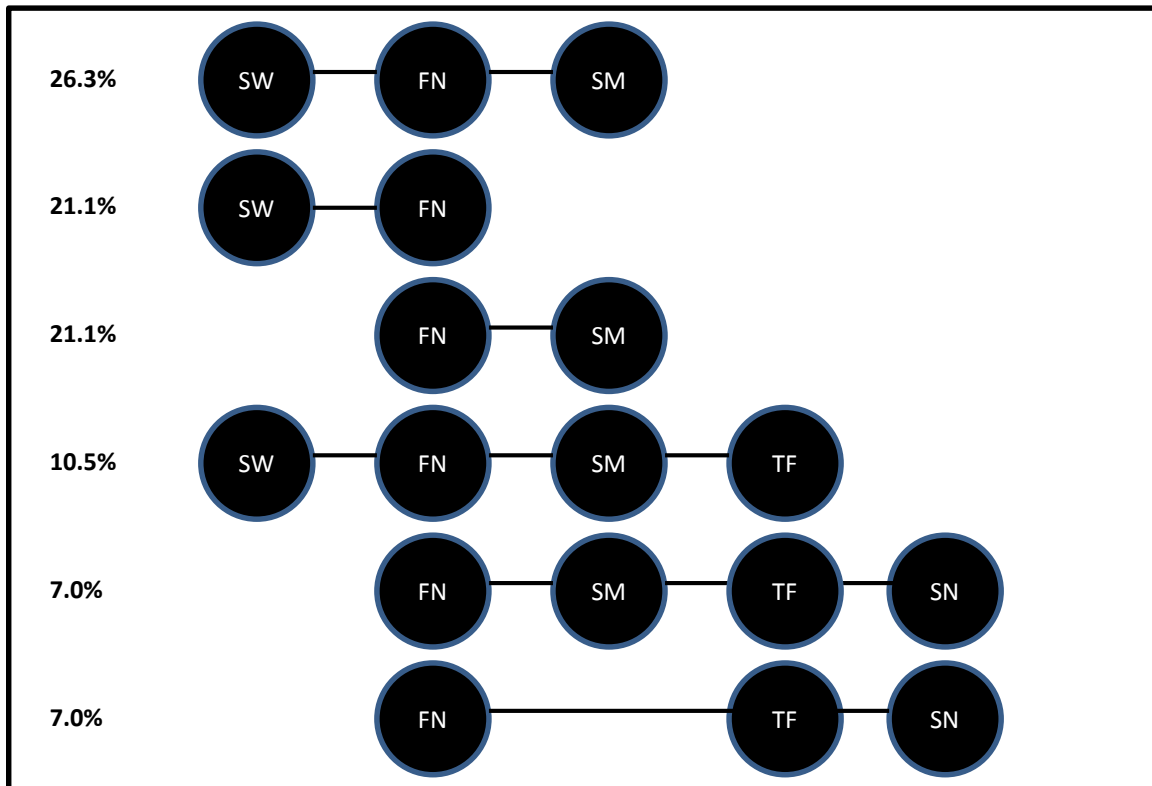


Figure 2. Comprehensive Microbial Oral Patient Sample Profile. Most samples harbored the microbial bridge species *F. nucleatum* (FN), which was often found in combination with other oral pathogens. The cariogenic pathogens *S. wiggsiae* (SW) and *S. mutans* (SN) were found in combination with FN in approximately one-fourth of samples analyzed, and were also found to be present concomitantly in another subset (26.3%). Other organisms, such as *T. forsythia* (TF), were found in smaller subsets, while *S. noxia* (SN) was only found among the SW-negative samples.

Conclusion

These data may suggest that *S. noxia* and *S. wiggsiae* may occupy distinct, non-overlapping niches, which may differ significantly from the interactions observed with *F. nucleatum*. The limited numbers of studies available regarding *S. wiggsiae* prevalence have suggested that *S. wiggsiae* and *S. mutans* may inhabit similar and overlapping niches within the oral microbiome. In fact, studies now suggest the potential for both competition and interactive inhibition between these organisms within the oral cavity. The preliminary data from this pilot study suggest *S. mutans* and *S. wiggsiae* may, in fact, be present in some of the same patients and may not therefore be exclusively competitive – at least in this patient population. However, due to the large differences observed among these samples, further research will be needed to more fully elucidate these interactions and to explore the potential ramifications for oral microbial ecology and the implications for predictive saliva screening.

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Chapter 5

Summary and Conclusions

As caries continues to remain widespread among the general population, being able to prevent the disease is equally as important as finding a way to treat it. Understanding the prevalence and ecology of cariogenic pathogens will help clinicians and oral healthcare providers better understand disease prevention and treatment. To date, there are a few studies on the novel cariogenic pathogen *Scardovia wiggisiae* and even fewer on its prevalence with other cariogenic pathogens. For this reason, this study's initial focus was to assess the prevalence of oral microbial flora in SW-positive and negative patients.

The first manuscript titled "Oral Microbial Ecology Of *Selenomonas Noxia* and *Scardovia Wiggisiae*" describes the ecology between SW and SN, two oral pathogens associated with poor oral health. The data suggests SN and SW may occupy distinct, non-overlapping niches within the oral microbiome and may be exclusively competitive. While SN and SW may not coinhabit the same niche, the interactions between SW, AA, FN, and TF show that they can. AA was only present among SW-positive samples, while FN and TF were present in both SW-positive and negative samples with variable prevalence. Previous studies have shown a strong correlation between SW and advanced early childhood caries. If treatment can change the oral microbiota in favor of SN, the treatment of dental caries may be improved.

The second manuscript titled "Screening a Saliva Repository for *Scardovia wiggisiae* and *Streptococcus mutans*: A Pilot Study" shows that SW and SM may inhabit similar and

overlapping niches within the oral microbiome. SM exists in the absence of SW, but in lower numbers. Similarly, SW can thrive in the absence of SM. This data suggests that SM and SW may not be exclusively competitive, but rather commensal organisms.

The third manuscript titled “Microbial ecology of *Scardovia wiggisiae*-positive and negative samples” analyzed the microbial pathogens in patient saliva screenings. FN was present in 93% of samples. SW (47%) and SM (40%) were present in nearly half of the patient samples. TF (29%) and SN (21%) were found in approximately one-quarter of the patient samples. SW, SM, and FN were found to coinhabit the same niche in approximately one-fourth of the samples analyzed.

This study helps to gain novel insight on the prevalence of specific microbiota in the oral cavity. Although far from preventing or treating oral disease completely, the data presented suggests a thorough understanding that will aid clinicians and healthcare providers in disease prevention and treatment. There is much more to be discovered about the epidemiology of cariogenic microbial pathogens, their interactions, and potential ecology.

Research Questions

1. Does the prevalence of cariogenic microbial flora vary in SW-positive patients?

H₀: The prevalence of cariogenic microbial flora in SW-positive patients is similar.

H_A: The prevalence of cariogenic microbial flora in SW-positive patients is different.

The null hypothesis is rejected because the prevalence of cariogenic microbial flora in SW-positive patients is different.

2. Does the prevalence of SM vary in SW-positive and negative patients?

H₀: The prevalence of SM in SW-positive and negative patients is similar.

H_A: The prevalence of SM in SW-positive and negative patients is different.

The null hypothesis is rejected because the prevalence of SM in SW-positive and negative patients is different.

Limitations and Recommendations

As one of the first studies to describe the interactions and potential ecology between oral microbial pathogens, there are some limitations that should be considered for future studies. First, our patient selection consisted of subjects who were patients of record at a public dental school with the majority of patients coming from low-income families and low socioeconomic backgrounds. This predisposes the patient pool to a bias among patients with higher caries risk and similar ethnic backgrounds. Both pediatric and adult patients were also used for this study, therefore no age specific information was provided. Secondly, because of the retrospective nature of this study, caries risk level was not documented. The variance in caries risk levels (low, moderate, and high) may affect the prevalence of specific cariogenic pathogens directly. Lastly, the storage of the saliva samples varied significantly which may have affected the quality and quantity of DNA isolated.

To continue elucidating the prevalence of oral microbial organisms, future studies can obtain new saliva samples from age specific patients, different ethnic backgrounds, and of different caries risk levels. Because orthodontic treatment changes the caries risk levels in individuals, studies can document the prevalence of specific organisms pre-, during, and post- orthodontic treatment to gain a better understanding between caries risk and microbial prevalence. Future studies can also include treatments done in the home such as chlorhexidine rinses, baking soda remedies, and toothpaste aids to observe their effect on microbial prevalence before and after treatment. If one treatment is more effective than another in changing the ecology and prevalence of specific organisms, then healthcare providers and clinicians may help treat oral diseases by placing greater emphasis on specific techniques during oral hygiene instruction.

Appendix A



UNLV Biomedical IRB - Exempt Review Exempt Notice

DATE: March 4, 2016

TO: Kari Kingsley, PhD, MPH
FROM: Office of Research Integrity - Human Subjects

PROTOCOL TITLE: [875879-1] Selenomonas Noxia prevalence in DNA previously isolated from pediatric patient saliva samples.

ACTION: DETERMINATION OF EXEMPT STATUS
EXEMPT DATE: March 4, 2016
REVIEW CATEGORY: Exemption category # 4

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 45CFR46.101(b) and deemed exempt.

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

PLEASE NOTE:

Upon final determination of exempt status, the research team is responsible for conducting the research as stated in the exempt application reviewed by the ORI - HS and/or the IRB which shall include using the most recently submitted Informed Consent/Assent Forms (Information Sheet) and recruitment materials. The official versions of these forms are indicated by footer which contains the date exempted.

Any changes to the application may cause this protocol to require a different level of IRB review. Should any changes need to be made, please submit a **Modification Form**. When the above-referenced protocol has been completed, please submit a **Continuing Review/Progress Completion report** to notify ORI - HS of its closure.

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.

Office of Research Integrity - Human Subjects
4505 Maryland Parkway . Box 451047 . Las Vegas, Nevada 89154-1047
(702) 895-2794 . FAX: (702) 895-0805 . IRB@unlv.edu

Appendix B

UNLV
Biomedical IRB
Notice of Excluded Activity

DATE: February 6, 2015
TO: **Dr. Karl Kingsley**, School of Dental Medicine
FROM: Office of Research Integrity – Human Subjects
RE: Notification of IRB Action
Protocol Title: **The Prevalence of Oral Microbes in Saliva from the UNLV School of Dental Medicine Pediatric and Adult Clinical Population**
Protocol# 1502-5068M

This memorandum is notification that the project referenced above has been reviewed as indicated in Federal regulatory statutes 45CFR46.

The protocol has been reviewed and deemed excluded from IRB review. It is not in need of further review or approval by the IRB.

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

If you have questions or require any assistance, please contact the Office of Research Integrity – Human Subjects at IRB@unlv.edu or call 702-895-2794.

Appendix C



**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 Wiggisiae* (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 D

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Microbiology Research Journal International

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Oral Microbial Ecology of *Selenomonas noxia* and *Scardovia wiggsiae*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AT, KK and KMH designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors SM and JM managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

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Original Research Article

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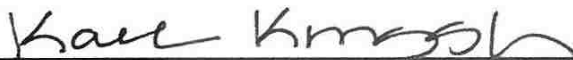
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Bacteriological and Histopathological Studies on Pulmonary Lesions of Camels (*Camelus dromedarius*) in Sudan

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This work was carried out in collaboration between all authors. Authors MEA and AMZ designed the study, wrote the protocol and histopathological studies. Author MEA collected samples, isolated and identify the bacteria and wrote the first draft of the manuscript. Authors MHS, AMA and MOH managed the literature searches and discussed the histopathological results. Authors IGI and HAMI revised the manuscript. All authors read and approved the final manuscript.

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Original Research Article

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Research Article

Microbial ecology of *Scardovia wiggisiae*-positive and negative samplesAmy Tam¹, Karl Kingsley^{2,*}

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Recent studies have reported a novel cariogenic pathogen *Scardovia wiggisiae* among patients with poor oral health. The prevalence of this organism in the context of additional caries risk factors, such as Orthodontic treatment has yet to be fully explored. In addition, few studies have evaluated the presence of other pathogenic organisms with respect to *Scardovia*-positive patients. Recent studies at this institution have revealed *S. wiggisiae* among orthodontic and non-orthodontic patients have generated sufficient information to briefly summarize and characterize the microbial ecology of *Scardovia wiggisiae*-positive and negative saliva samples within this patient population. In brief, the cariogenic pathogens *S. wiggisiae* and *S. mutans* can be found separately or in combination in approximately half of all patient samples. However, the presence of other organisms, most notably *Selenomonas noxia* and *Tannerella forsythia* were only found in the *Scardovia*-negative samples – which may suggest these organisms or other factors that promote their growth or aggregation may selectively inhibit *S. wiggisiae*.

Keywords: *Scardovia wiggisiae*, Pediatric, Adult Saliva Screening, Microbial Ecology

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