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Research Article

Evaluation of Blood Type and Oral Microbial Prevalence Suggests Potential Modulation of *Selenomonas noxia* but not *Scardovia wiggsiae* by Oral Antibodies: A Pilot Study

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Abstract

Introduction: The goal of this project was to screen an existing saliva biorepository to determine patient blood type (A, B, AB, O) for potential correlations with the presence (or absence) of oral microbial pathogens, including *Scardovia wiggsiae* (SW), *Selenomonas noxia* (SN), *Porphyromonas gingivalis* (PG), and *Streptococcus mutans* (SM).

Methods: A total of n = 50 samples from an existing saliva repository were screened for A-B-O blood groups revealing 58% were blood type O, 32% were blood type A, 10% were blood type B and none were blood type AB, which was not significantly different from the overall distribution of blood groups in the overall population (52.6%, 29.4%, 15.8%, 3.2%, respectively), p = 0.3652.

Results: All samples were screened for the presence of SW, SN, PG and SM, revealing 100% of SN-positives were among the A- (75%) and B- (25%) blood groups with no SN positive samples found among the O- (0%) blood group, p = 0.0629. PG-positives were equally split mostly between the O- (50%) and A- (50%) blood group with no PG-positive samples among the B- blood group (0%), p = 0.5290. In contrast, SM-positives were found among patients with A- (64%) B- (21%) and O- (14%) blood types, p = 0.6566. with SW-positives found among patients with O- (55%), A- (27%) and B- (18%) type blood, p = 0.6584.

Conclusion: These data suggest host modulation of the oral microbiome by anti-A and -B antibodies, which may affect both SN and PG periodontal pathogens. Both cariogenic pathogens SM and SW were mostly found among the O-blood group, suggesting anti-A and anti-B antibodies had limited or no specific effects. These data may represent one of the first studies to determine whether blood types and the resulting oral antibodies may be associated with oral prevalence of the newly characterized periodontal and cariogenic pathogens *Selenomonas noxia* and *Scardovia wiggsiae*, specifically.

Keywords: *Saliva Screening; Oral Microbial Pathogens; ABO Blood Groups; Oral Microbiome*

Introduction

The A-B-O blood group system and associated antibodies have been demonstrated to have a significant influence on microbial communities in the gastrointestinal tract, as well as the oral cavity [1,2]. For example, the antibodies specific to the A-B-O blood groups have

been demonstrated to mediate susceptibility to rotavirus and norovirus infection [3,4]. More recently, evidence has demonstrated that the A-B-O blood group antigens and host responses may have the potential to module other viral diseases, including the human immunodeficiency virus (HIV) and SARS-CoV-2 (COVID-19) infection [5-8].

In addition to the modulation of viral infections, the A-B-O blood group antigens and related antibodies are also known to influence the propensity for several bacterial-related diseases [9]. The susceptibility to, and severity of, infection from *Escherichia coli* has been demonstrated with A- and B-blood group antigens and their respective antibodies [10,11]. Moreover, research has demonstrated that infection and pathogenicity of *Salmonella typhi* may also be integrally involved with the A-B-O blood group system and host immune responses [12].

Most recently, research efforts have been highly focused on the relationship between A-B-O blood groups and oral disease, such as periodontal disease [13-15]. For example, studies have revealed the relationship between A-B-O blood group antibodies and caries development [16-18]. More specifically, these studies have revealed that patients with Blood Group O (with anti-A and anti-B antibodies) have the lowest decayed-missing- filled- teeth (DMFT) scores, while patients with Blood Group AB (with no anti-A or anti-B antibodies) have the highest DMFT scores. However, new evidence has revealed recently-discovered oral pathogens that may have as yet undefined associations with oral antibodies and A-B-O blood groups [19,20].

Two of these recently discovered oral pathogens are the periodontal-disease associated *Selenomonas noxia* (*S. noxia*) and the cariogenic bacteria *Scardovia wiggsiae* (*S. wiggsiae*), which have been evaluated by microbial screenings from this group [21,22]. More specifically, the gram-negative bacterium *S. noxia* has been linked not only with the development and progression of periodontal disease and caries in the oral cavity, but may also be an important modulator of obesity as part of the gastrointestinal microbiome [22-25]. The gram positive organism *S. wiggsiae* has been identified in caries lesions and patients with high-caries risk and has also been demonstrated to drive the development and progression of periodontal disease [26-28]. In addition, *Scardovia wiggsiae* has also been isolated from and strongly associated with severe early childhood caries (SECC) lesions among children and adolescents [29,30].

However, due to the recent nature of these discoveries, much less is known about the prevalence of these organisms, their oral habitats, and whether or not blood type and the antigen-antibody relationships observed with other oral bacteria apply to these organisms [31,32]. Based upon this lack of knowledge, the primary objective of this project was to utilize our clinical sample biorepository to determine if patient blood type correlates with the presence (or absence) of these specific oral microbial pathogens. The working research question and hypothesis is that *S. wiggsiae* and *S. noxia* prevalence may be modulated by blood group of the host, similar to observations made with more well-characterized periodontal and cariogenic pathogens, such as *Porphyromonas gingivalis* (*P. gingivalis*) and *Streptococcus mutans* (*S. mutans*).

Materials and Methods

Study protocol

This study was conducted according to the guidelines of the Declaration of Helsinki. and was reviewed and approved by the University of Nevada, Las Vegas (UNLV) Institutional review board (IRB) under protocol 1619329-1 titled "Retrospective analysis of Oral Health Status of Dental Population" on 24 July 2020. This retrospective analysis of previously collected samples was deemed Exempt pursuant to the Basic Health and Human Services (HHS) Policy for the Protection of Human Research Subjects (46.101) regarding IRB exemption for research that involves the study of existing data, documents or records that current exist and are not prospectively collected and 1. Participants cannot be directly identified; and 2. Participants cannot be identified through identifiers linked to them.

Using an existing saliva repository database, n = 878 potential samples were. Of these samples, n = 285 had been previously classified according to bacterial concentration. The samples with sufficient quantity of saliva were then screened for DNA concentration using a

NanoDrop 2000 spectrophotometer (> 10 ng) and purity (absorbance ratio at A260 nm compared with absorbance at A280 nm; ratio > 1.65). Samples with sufficient DNA concentration and purity were genotyped for A-B-O blood groups using allele-specific qPCR primers specific to the single nucleotide polymorphisms (SNPs) for each group at nucleotide positions 261, 769, and 803. A-B-O genotypes were compared against the previous microbial screening results to determine any potential relationships between A-B-O blood group and the presence or prevalence of the cariogenic pathogen *S. wiggsiae* or the periodontal pathogen *S. noxia*.

An additional cariogenic pathogen *S. mutans* and another periodontal pathogen (*P. gingivalis*) were also included for this analysis The categorical data, such as *S. wiggsiae* or *S. noxia*-positive or negative samples, were then compared using Chi square analysis.

Informed consent and original salivary sample collection protocol

The protocol utilized for the collection of samples of saliva was approved under UNLV IRB Office for the Protection of Research Subjects (OPRS) Protocol#1305-4466M "The Prevalence of Oral Microbes in Saliva from the UNLV School of Dental Medicine Pediatric and Adult Clinical Population" in 2013. In order to conduct the study, pediatric and adult patients were asked to provide consent or assent where applicable. Patients above the age of 18 who agreed to voluntary participation were asked to provide Informed Consent. Patients under the age of 18 but over the age of seven were asked to provide Pediatric Assent, as well as obtaining Informed Consent from the Parent or Guardian accompanying these patients. The primary goals of the original study (evaluation of the prevalence of oral microbes in saliva) were described to patients and all subsequent and follow up questions were answered. No remuneration was offered to any study participants. In addition, all patients were notified that any results from the study would not be connected with any patient identifying information - and based upon this type of study no potential notifications would be possible to alert them of specific oral bacteria found within any particular patient sample.

All samples were collected at the beginning of one of the randomly chosen clinic sessions, prior to any clinical treatment. All sample selection and clinical saliva collection dates were randomly chosen using a data algorithm that assigns a clinic session number (e.g. Monday March 1, AM patients = 1, Monday March 1, PM patient clinic = 2) with a set of randomly generated numbers to identify specific collection dates and times. Students and clinicians were then provided with the sample collection materials on the appropriate dates and times, which included sterile 50 mL polypropylene collection tubes, randomly generated labels and a basic intake information sheet that only provides basic demographic information, such as age of the patient, sex or gender as reported by the patient, and age of the patient at the date of the collection. Collection of each clinical sample included a maximum of 5.0 mL of saliva collected in polypropylene tubes, which were labeled using the previously mentioned randomized, non-duplicated numbers that were specifically generated to prevent the potential sharing of or linkage with patient-identifying information. All samples were transferred to a biomedical laboratory for long-term storage at -80C in a locked freezer.

DNA analysis and isolation

Each clinical sample of DNA was isolated with the method of phenol:chloroform extraction using a reagent from Thermofisher Scientific, TRIzol DNA isolation reagent (Fair Lawn, NJ). First, samples were taken out of the freezer, thawed and then placed in a sterile microcentrifuge tube to centrifuge 400 uL of saliva, with equal parts of TRIzol reagent. Then an additional 200 uL of chloroform was added and incubated for 10 minutes over ice. Using the Eppendorf Refrigerated Microcentrifuge, the tubes were spun at a rate of 12,000 x g at 15 minutes. After the centrifuging process was complete, the aqueous phase found in the upper aspect of the tube was moved to another sterile microcentrifuge tube with an equal volume of isopropanol to allow thorough DNA mixing. At this point, samples were centrifuged for another 10 minutes and the isopropanol was removed from the tubes. Samples were removed and washed with molecular grade ethanol and centrifuged for another 5 minutes. Then the ethanol was removed and samples were suspended with nuclease-free distilled water. Using the NanoDrop 200 Spectrophotometer from Fisher Scientific (Fair Lawn, NJ), the DNA was screened and readings of absorbance were

used at A260 nm and A280 nm to determine purity of samples of DNA. If a sample contained sufficient quantity, > 10 ng, and significant quality as determined by the A260:A280 ratio, > 1.65, the samples were chosen for qPCR screening.

qPCR screening

Using the QuantStudio Real-Time Polymerase Chain Reaction (PCR) system from Applied Biosciences (Waltham, MA), the samples were screened using specific primers. The forward and reverse primers (1.75 uL each), along with ABsolute SYBR Green (12.5 uL) SYBR Green, nuclease-free water (7.5 uL), and sample DNA (1.5 uL) diluted to 1.0 ng/uL, made up the 25 uL total reaction volume of qPCR Master Mix from ThermoFisher Scientific (Fair Lawn, NJ) that was utilized during the screening qPCR reactions. Each cycle was set at 95°C for 15 minutes with 40 cycles of denaturation at 95°C for 15 seconds each, followed by annealing at 30 seconds set at each primer's specified temperature, with final extension of 30 seconds at 72°C. The primer sets used for sequencing included the following:

O blood group primer set

Forward ABO-O primer: 5'-AGC TGT CAG TGC TGG AGA TGC-3'

Reverse ABO-O primer: 5'-TCC ACG CAC ACC AGG TAA TC-3'

A blood group primer set

Forward ABO-A primer: 5'-GAG ATC CTG ACT CCG CTG TT-3'

Reverse ABO-A primer: 5'-CCG ACC CCC CGA AGT ACC-3'

B blood group primer set

Forward ABO-B primer: 5'-AGG AAG GAT GTC CTC GTG TTA C-3'

Reverse ABO-B primer: 5'-GTT CTG GAG CCT GAA CTG CT-3'

Positive control, bacterial 16S rRNA primer set

Forward 16S rRNA primer: 5′-ACG CGT CGA CAG AGT TTG ATC CTG GCT-3′

Reverse 16S rRNA primer:5′-GGG ACT ACC AGG GTA TCT AAT-3

S. wiggsiae (SW) primer set

Forward SW primer 5'-GTG GAC TTT ATG AAT AAG C-3'

Reverse SW primer 5'-CTA CCG TTA AGC AGT AAG-3'

S. noxia (SN) primer set

Forward SN primer 5'-TCT GGG CTA CAC ACG TAC TAC AAT G-3'

Reverse SN primer 5'-GCC TGC AAT CCG AAC TGA GA-3'

S. mutans (SM) primer set

Forward SM primer 5'-GCC TAC AGC TCA GAG ATG CTA TTC T-3'

Reverse SM primer 5'-GCC ATA CAC CAC TCA TGA ATT GA-3'

P. gingivalis (PG) primer set

Forward PG primer 5'-TAC CCA TCG TCG CCT TGG T-3'

Reverse PG primer 5'-CGG ACT AAA ACC GCA TAC ACT TG-3'

Statistical analysis

Statistics describing demographic variables such as blood type, gender, race or ethnicity, age range, and clinic specific designations such as pediatrics or orthodontics, were organized in Microsoft Excel (Redmond, WA). Descriptive statistics were calculated for these categorical variables and compared with the overall clinic population statistics using Chi Square analysis, which is appropriate for categorical, non-parametric data analysis. Samples were screened and categorized into their respective blood types and then further divided into four categories: SW positive, SM positive, SW negative, and SM negative. The samples were analyzed using Chi Square statistical methods, which are commonly used for this type of non-parametric (categorical) data analysis. The significance level chosen for all calculations was alpha = 0.05.

Results

A total of n = 50 samples were identified from an existing saliva biorepository (Table 1). Analysis of the demographic characteristics from the study sample revealed that n = 31/50 or 62% of the patients were female, which approximates the percentage of females observed within the overall clinical population from other recent studies (55%), p = 0.1594 [21,22,31,33,34]. In addition, the percentage of minorities in the study sample (64%) very closely approximates the percentage of minorities observed within the overall clinical population (65%), p = 0.8338 [21,22,31,33,34]. Finally, the number of samples was balanced between adult and pediatric patients, as well as the percentage of patients with and without fixed orthodontic appliances (50% each).

Demographic	Study Sample	Clinic Population	Statistical analysis
Sex			
Male	n = 19/50 (38%)	45%	X^2 = 1.980, d.f. = 1
Female	n = 31/50 (62%)	55%	p = 0.1594
Race or Ethnicity			
White	n = 18/50 (36%)	35%	X^2 = 0.044, d.f. = 1
Minority (non-White)	n = 32/50 (64%)	65%	p = 0.8338
Patient clinic			
Pediatric	n = 25/50 (50%) Average age: 12.1 yrs Range 7 - 17 years		
Adult	n = 25/50 (50%) Average age: 42.3 yrs Range: 18 - 73 yrs.		
Orthodontic	n = 25/50 (50%)		
Non-Orthodontic	n = 25/50 (50%)		

Table 1: *Demographic analysis of study samples.*

DNA was isolated from both the pediatric and adult saliva samples and subsequently screened using spectrophotometric analysis to determine DNA quality and quality (Table 2). These data demonstrated that the average DNA concentration from pediatric saliva samples (average 518.2 ng/uL) was within the range specified by the manufacturer for isolation from biological samples (100 - 1000 ng/uL) and

was more than sufficient for qPCR screening. In addition, the quality of DNA as measured by the ratio of absorbance at A260 and A280 nm (A260:A280 ratio = 1.76 average) was also more than sufficient for qPCR as determined by the manufacturer protocol (A260:A280 ratio > 1.65). Similarly, the concentration (544.3 ng/uL) and quality (A260:A280 ratio = 1.75 average) of DNA from adult saliva samples was also found to be sufficient for qPCR screening and analysis.

Study sample	DNA concentration	DNA purity (A260:A280 ratio)
Pediatric samples (n = 25)	Average 518.2 ng/uL +/- 51.5 Range: 272.1 - 679.3 ng/uL	Average: 1.74 Range: 1.69 - 1.81
Adult samples (n = 25)	Average 544.3 ng/uL +/- 57.7 Range: 396.1 - 732.1 ng/uL	Average: 1.76 Range: 1.71 - 1.83

Table 2: *Analysis of DNA from study samples.*

Screening of the study samples revealed that a majority (n = 29/50 or 58%) were homozygous for the O/O blood group (Figure 1). In addition, a significant proportion of the study sample was found to have blood type A (n = 16/50 or 32%) consisting of homozygous A/A (n = 8/50 or 16%) or heterozygotes A/O (n = 8/50 or 16%). Finally, a small subset of samples were found to have blood type B (n = 5/50 or 10%), which were either homozygous B/B (n = 1/50 or 2%) or heterozygotes B/O (n = 4/50 or 8%). None of the samples tested were A/B blood type (n = 0/50 or 0%). These percentages from the study sample were not significantly different from the overall distribution of blood groups in the overall population, including the O blood group (52.6%), A blood group (29.4%), B blood group (15.8%), and AB blood group (3.2%), p = 0.3652.

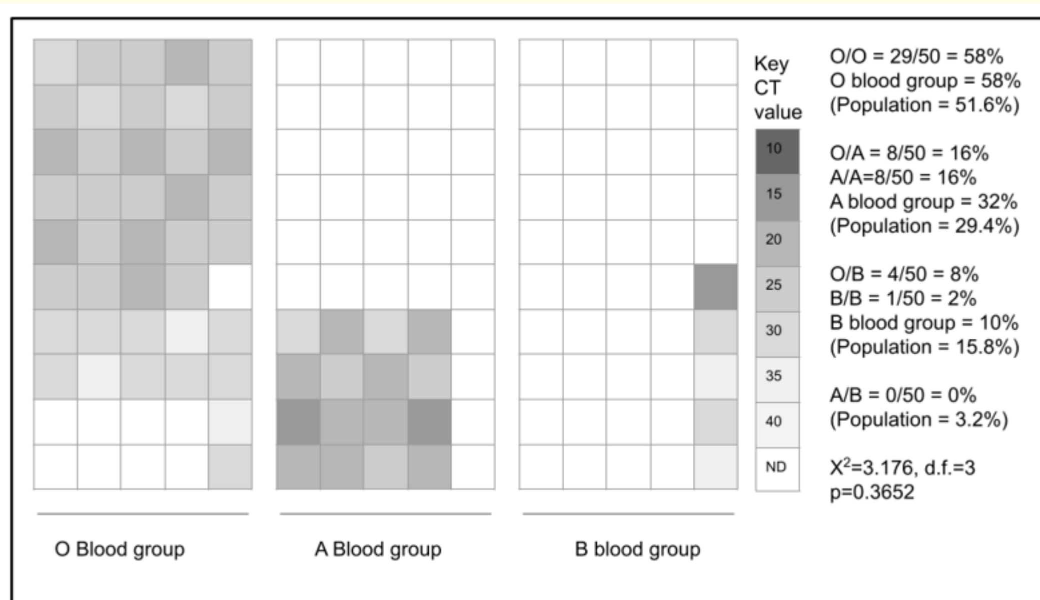

Figure 1: *qPCR screening of the study samples for blood type. The majority (n = 29/50 or 58%) were homozygous for the O/O blood group. Many samples were blood type A (n = 16/50 or 32%) consisting of homozygous A/A (n = 8/50 or 16%) or heterozygotes A/O (n = 8/50 or 16%) with a small number exhibiting blood type B (n = 5/50 or 10%), which were homozygous B/B (n = 1/50 or 2%) or heterozygotes B/O (n = 4/50 or 8%), which approximates the overall distribution of blood groups in the overall population: O blood group (52.6%), A blood group (29.4%), B blood group (15.8%), and AB blood group (3.2%), p = 0.3652.*

To evaluate whether patient blood type was associated with the prevalence of periodontal pathogens, qPCR screening for *S. noxia* and *P. gingivalis* was performed (Figure 2). These data demonstrated that patient samples harboring *S. noxia* (SN) were found exclusively among patients with A- (75%) and B-blood (25%) types. SN was not found among patients with the most common type O blood, which was different than would be expected if oral antibodies associated with blood groups did not mediate their distribution but was not statistically significant (p = 0.0629). In addition, these data revealed that samples harboring *P. gingivalis* (PG) were found among both O- (50%) and A-type (50%) blood, but not among patients with type B blood, which was also different than would be expected - although the small number of patient with type B blood in this sample may limit the inferences that can be made in this pilot study, p = 0.5290.

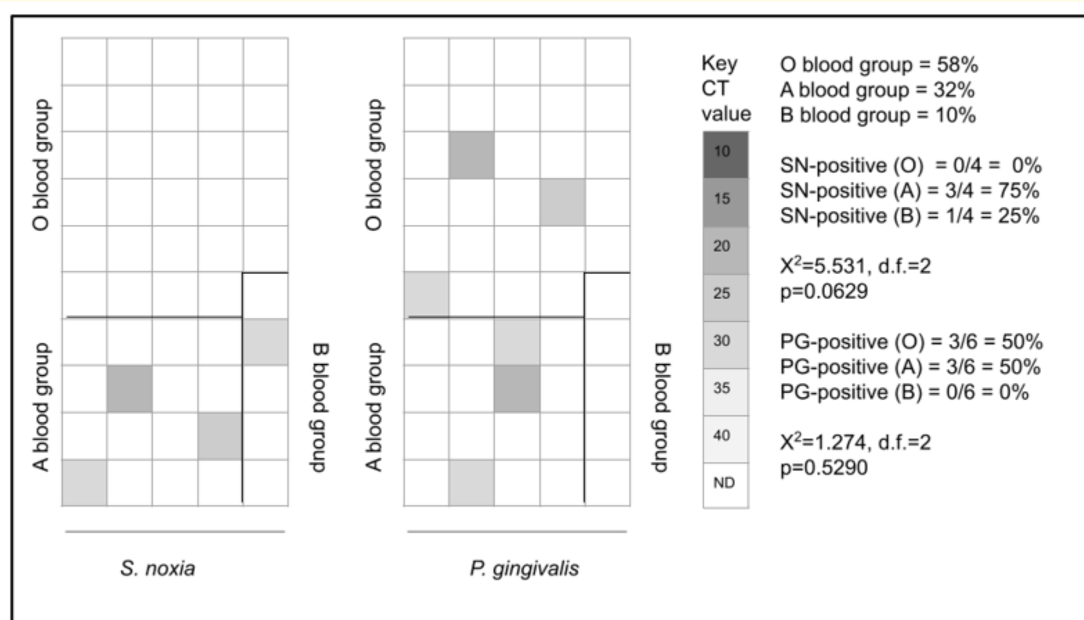

Figure 2: *qPCR screening for periodontal pathogens. qPCR sample screening for S. noxia (SN) revealed positives among patients with A- (75%) and B- (25%) blood types but not type O blood, p = 0.0629. In addition, P. gingivalis (PG) was found among patients with both O- (50%) and A- (50%) type blood, but not with type B blood, p = 0.5290.*

To evaluate any association of patient blood type with cariogenic pathogens, qPCR screening was performed using primers for *S. mutans* and *S. wiggsiae* (Figure 3). These data revealed that positive samples for *S. mutans* (SM) were found among the O- (64.3%), A- (21.4%), and B- (14.3%) blood types, which was not significantly different than expected based upon the distribution of blood types, p = 0.6566. In addition, positive samples harboring *S. wiggsiae* (SW) were also found among the O- (54.5%), A- (27.3%) and B- (18.2%) blood types, which was also not significantly different than expected based upon the overall distribution of blood types, p = 0.6584.

Discussion

The primary objective of the study was to evaluate any association between blood type (group) and the presence of the more recently identified pathogens *S. noxia* and *S. wiggsiae*. Although some previous studies have demonstrated that blood groups may correlate with cariogenic pathogens, such as *S. mutans*, this study found no association between blood type and the presence of *S. mutans.* This may be explained, in part, by previous research that has revealed that the high prevalence of high-risk, low-income patients at UNLV-SDM is as-

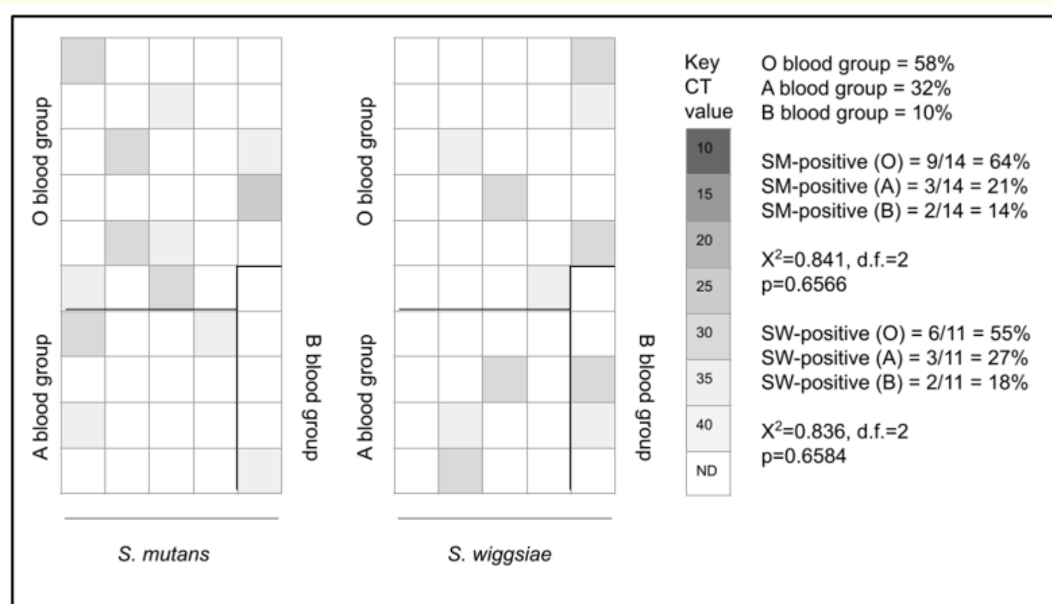

Figure 3: *qPCR screening for cariogenic pathogens. qPCR sample screening for S. mutans (SM) revealed positives among patients with A- (64%) B- (21%) and O- (14%) blood types, p = 0.6566. In addition, S. wiggsiae (SW) was found among patients with O- (55%), A- (27%) and B- (18%) type blood, p = 0.6584.*

sociated with greatly increased decayed-missing-filled teeth (DMFT) scores [35,36]. Although no previous research has yet evaluated the association of blood type with *S. wiggsiae*, these results also demonstrated that this pathogen was also not significantly associated with blood type, although these results may be influenced, in part, by the patient demographics and significant health challenges that face this specific patient population [37,38].

However, these results demonstrated that significant differences were found among the prevalence of periodontal pathogens *P. gingivalis* and *S. noxia* and patient blood types. More specifically, these data demonstrated that no patients with blood type O were found to harbor *S. noxia*, a novel and potentially important clinical finding. Although periodontal pathogens are typically found in the gingival crevicular fluid, recent observations have also detected this pathogen harboring in additional oral sites, such as biofilms on the dorsum of the tongue and supragingival tooth plaque [21,39]. As a gram-negative secondary or tertiary colonizer in biofilm, these locations could potentially render this organism susceptible to the effects of oral anti-A and anti-B blood group antibodies redefining how this constituent of the oral microbiome may be modulated by host immunity [40].

In fact, if these observations can be confirmed within the broader population, the potential may exist to develop oral immunizations to limit the potential colonization by this organism [41]. This may have significant implications for oral and systemic health, as *S. noxia* was identified as a potential direct contributor to obesity and has been a reliable biomarker for overweight and obesity due to the metabolic potential to extract calories from indigestible fibers and cellulose [42]. In fact, some studies of dietary modification have also found changes in the oral and gastrointestinal microbiome with a significant reduction in the levels of salivary periodontal pathogens among these patients - providing further evidence for the clinical relevance of the current study [43].

In addition, this study also found another association between the lack of detectable *P. gingivalis* among patients with type B blood. Although this represents a small pilot study, which must be confirmed in larger community-based studies - it may be the first evidence of this association and the potential for oral anti-A antibodies to modulate oral prevalence of this organism [44]. One previous study found that use of capsular polysaccharide from *P. gingivalis* (eliciting IgM responses only) was associated with production of antibodies that improve clinical symptoms of disease in a murine model [45].

Despite the significance of these findings, there are some limitations that should also be considered when reviewing these results. First, this was a small, pilot study that utilized an existing salivary biorepository from a publicly-funded dental school clinical to screen for the association between blood groups and newly identified oral pathogens. Furthermore, the patient population has significant oral health challenges and access to care, which may not be representative of the broader population at large [35-38]. In addition, due to the limitations of the original protocols for the collection of saliva samples - many other confounding factors are unknown, such as the oral health status, periodontal pocket depth (PPD), and DMFT scores of these patients, which could be included in future prospective studies that follow. Furthermore, this retrospective study was limited to a very small number due to the small sample size of the remaining samples within the biorepository. Finally, the current study did not have sufficient biorepository materials to conduct an analysis of the secreted antibodies that may have been responsible for some of these observations, which might be separate and distinct from the IgG-mediated mechanisms associated with periodontal disease and gingival crevicular fluid [46,47]. Other retrospective studies that seek to evaluate samples included in biorepository materials or additional future prospective studies of this nature may include this type of analysis, which would provide more definitive and clinically applicable results.

Conclusion

In summary, this pilot study provides some preliminary evidence for an association between some oral pathogens and patient blood types, which may be sufficient to spur further research in this area. In addition, future studies that have the potential to incorporate larger sample sizes and more patients with the less common blood types, such as B and AB, may be helpful to further our understanding of these observations. These findings suggest that further investigation may be needed to explore any potential mechanisms and other associations that might help to improve oral health and prevent oral and systemic disease.

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Author Contributions

KK was responsible for the overall project design. TP, RS, and KK were responsible for data generation. KK was responsible for analysis and the writing of this manuscript.

Conflict of Interest

The authors declare that they have no conflicts of interest to report.

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