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POSTGRADUATE DENTAL COLLEGE
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THESIS APPROVAL PAGE FOR MASTER OF SCIENCE IN ORAL BIOLOGY

Title of Thesis: “Preventative Strategies for Dental Caries using Phage Therapy”

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Master of Science
14 May 2021

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Preventative Strategies for Dental Caries using Phage Therapy

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Research satisfies AFDS Capabilities-Based Assessment #4
and USU Operational Gap IV, B

Abstract

The treatment of dental caries is progressing towards preventative measures. Current modalities include patient education and the use of fluoridated communal water supplies. A new preventative strategy is being researched to treat a different aspect of caries prevention- targeted viral therapy. Infection of *Streptococcus mutans* with bacteriophages, which causes bacteria to lyse and thereby halts the caries progression, is being researched. **Objective:** The purpose of this study was to discover naturally occurring bacteriophages that target *S. mutans*, the bacteria responsible for the initiation of caries. **Methods:** Clinical plaque and saliva samples were collected from 135 subjects. Plaque samples were placed in vials containing AS-920 or DNA/RNA Shield, while saliva samples were placed in vials of AS-920 or OMNIgene Oral DNA collection kit. Both samples were frozen at -80 degrees centigrade and kept frozen until analysis. 16S metagenomics sequencing was conducted using MiSeq Illumina. Attempts at phage induction and subsequent isolation was performed using various concentrations of mitomicin-C. **Results:** *S. mutans* was among the top eight bacterial species within thirteen of the plaque samples, with percentages ranging from 0.98-20.39% of the total bacterial species present. For a subset of samples, *S. mutans* was successfully isolated and underwent whole genome sequencing, revealing that bacteriophage DNA was incorporated into the genome of eleven samples. Short read analysis was completed on 17 samples, yielding *S. mutans* ranging from 0-27.51%, and streptococcus phage ranged from 0-0.45%. Samples with confirmed presence of phage ranged from 1-11 phages, with a percentage range of 0-0.38%. Efforts to

elicit bacteriophage from host strains of the thirteen plaque and saliva samples confirmed to contain *S. mutans* did not yield any bacteriophage. **Conclusion:** Bacteriophages may be used as a plausible clinical treatment modality for future caries prevention strategies, however discovery and isolation are obstacles that may prevent or hinder the clinical application of phage therapy.

Keywords: Bacteriophage, bacterial plaque, dental caries, caries prevention

INTRODUCTION

Dental caries is recognized as a frequently occurring disease by the CDC, with 91% of adults having experienced dental caries [1].Within the military population, it represents the greatest single threat to dental readiness, accounting for 39% of 40,000 dental disease and non-battle injuries studied in Iraq and Afghanistan from 2009 to 2012 [2, 3]. It is one of the most common diseases in military recruits [3]. The consequences of dental caries are frequently painful, distract from the mission, and are costly to treat [4]. Dental caries is preventable, as are half of military dental emergencies [2]. Unfortunately, prevention relies on patient compliance, which is known to be poor [5] and is often neglected in stressful operational or training environments [3, 5].

The cariogenic process starts in the oral cavity when bacteria create a biofilm, which provides them with a protective environment to thrive. Biofilms are bacterial communities that are surrounded and held to their substrate (the tooth) by an exopolysaccharide matrix. *Streptococcus mutans* is a keystone species in the polymicrobial plaque biofilms. This bacteria produces surface adhesives which interact with the pellicle present on teeth, initiating biofilm formation. Other bacteria, such as *Lactobacilli* spp. and *Actinomycetes* spp., will further colonize the biofilm and ultimately produce cellular products that lead to dental caries [6,7]. As the bacteria begin further colonizing the surfaces of teeth, their cellular products drop the pH below 5.5 - the threshold at which enamel demineralization occurs. Saliva is a natural defense to combat this, as it provides minerals along with raising the pH to allow teeth to remineralize. However, with the consumption of sucrose

[8], the process of remineralization and demineralization becomes unbalanced, leading to bacterial invasion further into the tooth. Once bacteria penetrate far enough into dentin, restorative measures must be taken. This caries process could not occur without *S. mutans*, however *Lactobacillus* spp., *Actinomyces* spp., as well as several other species of *Prevotella* and *Fusobacterium* have been found in deep carious lesions [9, 10, 11, 12].

The current modalities of treating dental caries are either reactive or proactive measures. In reactive measures, caries are removed and a bio-compatible material replaces missing tooth structure. Proactive measures, on the other hand, aim to address caries before the tooth reaches the point of needing repair. Recently, dentistry has been shifting its treatment focus towards preventative measures, to include proper patient education, communal fluoride programs, and placement of sealants on caries-prone surfaces [13]. Although these proactive treatment modalities are effective in preventing caries, they rely heavily on patient compliance, can be technique sensitive, and all focus only on the teeth opposed to addressing other aspects of the decay process. Instead of looking at ways to fortify teeth to prevent caries, targeting the bacteria may provide a new treatment option [14].

A proposed but unrealized therapy for controlling biofilm in the oral cavity is bacteriophage, or phage, therapy. This cellular level strategy is applied to bacteria via viruses using lytic or lysogenic pathways. Both methods have cellular invasion by the virus, however the difference between the cycles lies in their effect on host cells. In the lytic stage, a virus invades and takes over host cell functions in order to further replicate. The host eventually becomes saturated with new phages, and will lyse which releases the new phages to invade other cells. On the other hand, a temperate phage in the lysogenic cycle will incorporate into their host's genome and influence the expression of cellular products, however rarely kill their host. The temperate phage may inhibit specific cell products that contribute to the breakdown or destruction of other structures, such as teeth. Bacteriophages were first discovered in 1915 [15, 16], however with the discovery and efficacy of antibiotics [17, 18, 19], they have been a relatively untouched area especially in dental

research. Due to the over prescription, misuse, and reliance of antibiotics, their efficacy has decreased, leading to super bugs: bacteria immune to antibiotic therapy [20]. With concerns for antimicrobial resistance, phage therapy has begun to be investigated once again as a viable therapy for microbial control. As of May 2021, four phages [15, 21, 22, 23, 24] have been identified to show effectiveness in reducing *S. mutans* via cell lysis: M102 [21], M102AD [22], ϕAPCM01 [23], and recently discovered SMHBZ8 [24].There is currently limited research on temperate (lysogenic) phages that affect bacteria involved in the caries process, and even more specifically *S. mutans*. With limited established phage research available, our study aimed to address three objectives. Our first goal was to collect clinical samples of oral pathogens, with emphasis on *S. mutans*. With the collected samples, our second goal was to then screen for phages that can infect those pathogens, and finally our third goal was to characterize them for potential therapeutic use. By identifying such phages, a therapeutic approach to inhibiting microbial byproducts can be developed resulting in decreased biofilm formation and ultimately dental caries. Our null hypotheses therefore were: 1) there would be few or no phages against S. mutans in clinical plaque and saliva samples, and 2) infection with phage(s) does not prevent or slow the initiation or progression of dental caries.

METHODS

The Institutional Review Board at Wilford Hall Ambulatory Surgical Center, Joint-Base San Antonio, Lackland, Texas approved this protocol (#FWH202000016H). Eligibility requirements included active duty military members, Department of Defense (DoD) and non-DoD beneficiaries, between the ages of 18-54 years old. Exclusion criteria included pregnancy and antibiotic or antiviral treatment within the last 6 months. A total of 135 patients were consented, and clinical plaque and saliva samples were collected. Plaque samples were collected using a sterilized curette, and placed in vials of pre-reduced, anaerobically sterilized liquid dental transport medium (AS-920, Anaerobe Systems, Morgan Hill, CA) and 500 µL vials containing DNA/RNA Shield for

genomic analysis (MiSeq, Illumina, San Diego, CA). One to three ml of saliva were then collected into a 5 mL microcentrifuge tube (Earthox, Millbrae, CA). The saliva sample was then partitioned into two vials using single-use sterile pipettes, and placed into sterilized liquid dental transport medium (AS-920) or a vial containing the OMNIgene Oral DNA collection kit (DNA Genotek, Ottawa, Ontario, Canada). All vials were disinfected with a single use germicidal wipe (Sani-Cloth, Professional Disposables International, Orangeburg, NY) prior to being placed in a transport container. Samples were transported to the research facilities within 48 hours of being collected to ensure adequate survival of bacteria. OMNIgene samples were held at room temperature until DNA extraction and isolation, while AS-920 and DNA/RNA shield samples were frozen at -80 degrees centigrade (Thermo Fisher, Waltham, MA) and kept frozen until thawed for phage isolation.

Two research facilities collaborated in order to perform various aspects of sample processing. Clinical Investigations and Research Support (CIRS, Wilford Hall Ambulatory Surgical Center, JBSA – Lackland, TX) completed 16S metagenomics sequencing on both plaque and saliva samples. Naval Medical Research Unit San Antonio (NAMRU-SA, JBSA-Ft. Sam Houston, TX) isolated S. mutans from clinical samples, and then screened for bacteriophages. CIRS additionally performed DNA analysis on putative S. mutans strains isolated from clinical samples by NAMRU to screen for bacteriophage integration into the bacterial genome.

Metagenomics sequencing was completed using a genomic sequencing instrument (MiSeq Illumina, San Diego, CA) at CIRS. Saliva in OMNIgene ORAL microbial DNA tubes was potentially infectious and was heat inactivated by incubating tubes at 50°C for 1 hour in a water bath. Inactivated saliva was divided into three, 500 µL aliquots and stored at -80°C until DNA extraction.

The sequencing process began with taking collected plaque and saliva samples and extracting microbial DNA using DNA micoprep kit (ZymBIOMICS, Irvine, CA) following manufacturer’s instructions. Extracted DNA quantity and quality was determined by fragment analysis using the High Sensitivity NGS Fragment Analysys Kit (Agilent, Santa Clara, CA). 7.5 µL of extracted DNA was

used as the starting amount for plaque and saliva samples. The first round of Amplicon PCR was used to amplify the 16s V3/V4 region using Kapa HiFi HostStart Ready Mix (Roche, Banford, CT) using the primers (figure 1) at 10 µM for 95°C for 3 min; 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds, & 72°C for 30 sec; 72°C for 5 minutes.

Forward Primer= 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG
Reverse Primer=5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

Figure 1. Forward and reverse primers for PCR of 16s V3/V4 region.

Once samples had completed PCR, they underwent magnetic bead purification using AMPure XP beads (Beckman Coulter, Brea, CA) to remove any additional primers or DNA fragments. PCR products were analyzed by fragment analysis to determine if products were the expected size of approximately 550 base pairs. A second round of PCR to add sequencing indices and adaptors was carried out using the cleaned PCR product as a template and Nextera XT Index Kit v2 (Illumina, San Diego, CA) at 95°C for 3 min; 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds, & 72°C for 30 sec; 72°C for 5 minutes. A second round of magnetic bead purification was then completed for removal of remnant DNA tags, and quality controlled to verify expected library amplicon size of approximately 630 base pairs. Sequencing libraries were then diluted to 4 nM using 10 mM Tris-HCl, pH8 and then multiplexed into equimolar amounts, denatured, and diluted to a final loading concentration of 8-10 pM following the Illumina 16S Metagenomic Sequencing Library Preparation guide. Sequencing was completed by using 2x300 base pairs with MiSeq Reagent kit v3 (600-cycle). Accuracy of samples were verified by completing two runs in the MiSeq system, and were required to meet minimum quality reads of 10,000 per sample. Quality of results were further verified by comparing genomic sequences to known bacterial specimens provided from ZymoBIOMICS (Zymo Research Corporation, Irvine, CA). A report based on the amplified V3 and V4 regions was generated from a database of 16s rRNA data, and classified the top 8 bacterial species by kingdom, phylum, class, order, family, genus, and species.

Isolation of *S. mutans* was completed by NAMRU. Once thawed, samples contained within AS-920 vials were homogenized by repeated pipetting and

vigorous shaking and vortexing. Homogenized samples were then diluted to various concentrations: 1:1 in PBS, 1:10 in PBS, and 1:100 in PBS (Phosphate-buffered saline). Isolation of individual colonies of *S. mutans* was then completed using quad-streaking via 20 µl and 100 µl pipettes and placed on various growth medias to determine optimal growth conditions. Colonies were selected and re-plated as stabs onto fresh media, and repeated as needed to confirm they were single isolates. Colonies were grown as liquid cultures and characterized on a microbial identification system (Biolog, Hayward, CA). Eight strains of putative *S. mutans* along with the 13 confirmed plaque samples containing *S. mutans* were then given to CIRS for whole genome sequencing.

250 ng of DNA isolated from purified bacterial colonies was prepared for whole genome sequencing to determine if bacteriophage DNA was incorporated into the bacterial genome using the Illumina Nextera DNA Flex Library prep kit with Nextera DNA CD Indexes (Illumina, San Diego, CA). Libraries were treated with Free Adaptor Blocking Reagent prior to sequencing on the NextSeq 500 System at 2 x 75 base pairs using a NextSeq High Output 150 cycle kit (Illumina, San Diego, CA).

Short reads to confirm results from NextSeq analysis and presence of viral genomes within bacterial samples was also completed using Metagenomics App v1.0.0 (Illumina, San Diego, CA). Kraken summary reports listed the predominant bacterial species present in each sample, based on number of reads mapped to the reference genome. Additionally, the reports lists likely bacteriophage candidates with reads mapped to phage reference genomes.

For bacteriophage screening, isolated *S. mutans* strains were grown into lawns by double agar overlay. Drop tests of saliva or homogenized plaque samples were added to freshly inoculated bacterial lawns. Varying concentrations of the antibiotic Mitomycin-C (1.5, 0.5, 0.3 µg/ml) were used to try and elicit phages from host cells.

RESULTS

From the 135 clinical plaque and saliva samples from patients, thirteen samples were determined to contain *S. mutans* as the top 8 species ranging in percentages from 0.98-20.39% of total bacterial species present (table 1).

Table 1. Samples containing S. mutans as top 8 bacterial species within sample

Sample Number	S. mutans Ranking (X/8)	Percentage
122	3	20.39%
9	2	18.30%
114	2	15.24%
42	2	15.10%
94	5	6.33%
38	4	5.77%
47	5	5.13%
43	4	4.53%
87	7	2.01%
100	8	1.80%
39	8	1.34%
116	8	1.19%
8	8	0.98%

13 of the 135 clinical plaque samples collected contained *S. mutans* as the top 8 species precent within the sample. Percentage of *S. mutans* ranged from 0.98% to 20.39%

The isolated S. *mutans* strains were genomically sequenced in addition to the eight putative *S. mutans* strains and determined elements of bacteriophage DNA were incorporated into at least one of the samples, however the percentage present was below computation level due to the scarcity of genome present within the sample.

The 21 isolated *S. mutans* strains (8 putative isolated strains and 13 confirmed S. mutans samples), did not yield any bacteriophage with attempts to elicit bacteriophages from host bacteria (table 2).

Table 2. Samples containing confirmed *S. mutans* treated for phage induction.								
Sample #	No Treatment		mitomycin-C (1.5 ug/mL)		mitomycin-C (0.5 ug/mL)		mytomycin-C (0.3 ug/mL)	
	Saliva	Plaque	Saliva	Plaque	Saliva	Plaque	Saliva	Plaque
8	X	X	X	X	X	X	X	X
9	X	X	X	X	X	X	X	X
37	X	X	X	X	X	X	X	X
38	X	X	X	X	X	X	X	X
42	X	X	X	X	X	X	X	X
43	X	X	X	X	X	X	X	X
48	X	X	X	X	X	X	X	X
87	X	X	X	X	X	X	X	X
94	X	X	X	X	X	X	X	X
100	X	X	X	X	X	X	X	X
114	X	X	X	X	X	X	X	X
116	X	X	X	X	X	X	X	X
122	X	X	X	X	X	X	X	X
Attemps to elicit phage from clinical plaque and saliva samples with known presence of S. mutans did not yield any phage.								

Whole genome sequencing completed on NextSeq did not yield the bacteriophage incorporation within *S. mutans* genome, however when short read analysis was completed using sequencing, 11 samples yielded presence of streptococcus phage. The presence of *S. mutans* within samples ranged from 0-27.51%, and streptococcus phage ranged from 0-.0.45% (table 3). Samples with confirmed presence of phage ranged from 1-11 phages, with a percentage range of 0-0.38% (table 4).

Table 3. Short read analysis.			
Sample ID	Classification of Read	Number of Reads	Percentage
S9-A	Unclassified	100,570	7.15%
	S. mutans	158	0.01%
	Streptococcus Phage	6,532	0.45%
S8-A	Unclassified	86,355	5.84%
	S. mutans	86	0.01%
	Streptococcus Phage	3,534	0.24%
S8-E	Unclassified	102,613	5.93%
	S. mutans	37	0.00%
	Streptococcus Phage	4,594	0.20%
P8-4	Unclassified	8,838,504	15.50%
	S. Mutans	1,781	0.00%
	Streptococcus Phage	60,765	0.10%
P9-C	Unclassified	195,645	10.35%
	S. Mutans	1,587	0.08%
	Streptococcus Phage	1,073	0.06%
P9-E	Unclassified	182,690	9.89%
	S. mutans	1,630	0.09%
	Streptococcus Phage	654	0.04%
P6-F	Unclassified	44,746,515	96.39%
	S. Mutans	46	0.00%
	Streptococcus Phage	3	0.00%
S8-B	Unclassified	72,619	5.17%
	S. mutans	676	0.05%
	Streptococcus Phage	-	0.00%
S8-D	Unclassified	71,242	5.61%
	S. mutans	246	0.02%
	Streptococcus Phage	2	0.00%
S9-D	Unclassified	124,048	9.15%
	S. mutans	125	0.01%
	Streptococcus Phage	30	0.00%
S9-F	Unclassified	157,379	10.46%
	S. mutans	508	0.03%
	Streptococcus Phage	-	0.00%
P1-O	Unclassified	32,945,119	72.51%
	S. Mutans	10,614,553	23.36%
	Streptococcus Phage	1,682	0.00%
P1-R	Unclassified	19,902,865	74.67%
	S. Mutans	5,982	0.02%
	Streptococcus Phage	-	0.00%
P5-A	Unclassified	50,952,229	96.21%
	S. Mutans	94	0.00%
	Streptococcus Phage	1	0.00%
P5-D	Unclassified	29,596,105	96.30%
	S. Mutans	1	0.00%
	Streptococcus Phage	-	0.00%
P5-H	Unclassified	19,775,768	35.22%
	S. Mutans	16,293	0.03%
	Streptococcus Phage	-	0.00%
P6-B	Unclassified	80,110,476	96.41%
	S. Mutans	5	0.00%
	Streptococcus Phage	-	0.00%

Short read analysis was completed using Illumina Kraken metagenomics app v1.0.0. Presence of S. mutans within samples ranged from 0-27.5%, with streptococcus phage present at values ranging from 0-0.45%

Table 4. Phage classification within samples containing phages.

Sample ID	Classification of Read	Number of Reads	Percentage
P8-4	Streptococcus phage YMC-2011	40,563	0.07%
	Streptococcus phage 2972	3,498	0.01%
	Streptococcus phage ALQ13.2	1,719	0.00%
	Streptococcus phage 858	1,572	0.00%
	Streptococcus phage 5093	1,029	0.00%
	Streptococcus phage TP-778L	331	0.00%
	Streptococcus phage Abc2	173	0.00%
	Streptococcus phage O1205	4	0.00%
	Streptococcus phage TP-J34	1	0.00%
	Streptococcus phage EJ-1	1,726	0.00%
	Streptococcus phage 20617	10,149	0.02%
S8-A	Streptococcus phage YMC-2011	2,151	0.15%
	Streptococcus phage 2972	193	0.01%
	Streptococcus phage 858	192	0.01%
	Streptococcus phage 5093	157	0.01%
	Streptococcus phage ALQ13.2	145	0.01%
	Streptococcus phage TP-778L	4	0.00%
	Streptococcus phage Abc2	2	0.00%
	Streptococcus phage EJ-1	85	0.01%
	Streptococcus phage 20617	605	0.04%
S8-E	Streptococcus phage YMC-2011	2,742	0.16%
	Streptococcus phage 2972	242	0.01%
	Streptococcus phage 858	237	0.01%
	Streptococcus phage 5093	199	0.01%
	Streptococcus phage ALQ13.2	192	0.01%
	Streptococcus phage TP-778L	6	0.00%
	Streptococcus phage EJ-1	138	0.01%
	Streptococcus phage 20617	838	0.05%
S9-A	Streptococcus phage YMC-2011	5,413	0.38%
	Streptococcus phage 7201	155	0.01%
	Streptococcus phage Abc2	153	0.01%
	Streptococcus phage DT1	69	0.00%
	Streptococcus phage Sfi21	33	0.00%
	Streptococcus phage Sfi19	3	0.00%
	Streptococcus phage SM	1	0.00%
	Streptococcus phage 20617	705	0.05%
P1-O	Streptococcus phage YMC-2011	1,387	0.00%
	Streptococcus phage TP-778L	86	0.00%
	Streptococcus phage 2972	52	0.00%
	Streptococcus phage O1205	33	0.00%
	Streptococcus phage Sfi21	26	0.00%
	Streptococcus phage 20617	101	0.00%
P9-C	Streptococcus phage PH10	560	0.03%
	Streptococcus phage phi3396	513	0.03%
P9-E	Streptococcus phage phi3396	327	0.02%
	Streptococcus phage PH10	327	0.02%
S8-D	Streptococcus phage O1205	2	0.00%
S9-D	Streptococcus phage O1205	30	0.00%
P5-A	Streptococcus phage YMC-2011	1	0.00%
P6-F	Streptococcus phage YMC-2011	3	0.00%

11 samples contained Streptococcus phage, with P8-4 indicating the presence of 11 phages. Percentage of phage for all samples ranged from 0-0.45%

DISCUSSION

In a study completed by Dalmasso et al., out of 85 saliva samples, they were able to isolate one bacteriophage, resulting in a 1.1% discovery rate [23]. To date, only four bacteriophages have been isolated and identified targeting *S. mutans* [15, 21, 22, 23, 24]. Although collection of plaque and saliva samples was relatively simple, the meticulous and cumbersome efforts to isolate and grow S. mutans leads to decreased chance of discovery for novel bacteriophages. A possible strategy to increase the yield of targeted bacteria would be to be utilize enrichment cultures. Ahmed et al. in 2019 utilized enrichment cultures and attempted *S. mutans* phage isolation from thirty-eight clinical plaque samples; although they were unable to isolate phage, they were successful in isolating six strains, and used an additional twelve strains to grow enough bacteria to attempt phage isolation [25]. By growing *S. mutans* from clinical samples combined with known strains, any clinical bacterial samples possibly infected with phage would then be able to infect the newly introduced *S. mutans*. This would in turn increase the yield of bacterial samples infected with phages, which could then be further isolated for attempted phage induction and characterization. Beyond the laboratory procedures, the phage life cycle provides unique challenges for proper isolation in itself.

Bacteriophages work by infecting hosts via lytic or lysogenic cycles. The lysogenic cycle allows for the phage to survive via genomic integration and may affect cellular expression of host products, however the lytic cycle infects, replicates, and in turn kills the host via cell lysis. The lytic cycle may have attributed to the unsuccessful attempts to isolate *S. mutans*, due to the fact the virus had eliminated the species from samples. On the other hand, the lysogenic cycle, which still causes to some level in host cell death, may have contributed to the low percentages of *S. mutans* present in clinical samples collected. In our study, there was a low yield of the desired bacteria, which further made it difficult to have adequate samples to screen for phages. As previously discussed, adding enrichment cultures to our protocol may have mitigated the low bacterial yield (0-27.51%) and thus low phage percentages (0-0.45%).

Although samples from this study only showed evidence of streptococcus phage in eleven samples with percentages ranging from 0-0.45%, further genomic analysis can be completed to determine if the phages are lytic or lysogenic. Harada et.al, and Leiman and Shneider noted indicators of lysogenic versus lytic phage is the presence of three molecular components: terminase major, capcid proteins, and tailfibers [17, 26]. The already collected data could undergo further analysis of the identified phages, and could indicate whether collected samples were either lytic or lysogenic in nature.

Of the 135 samples collected, only 13 showed evidence of *S. mutans* to a quantifiable percentage. Although the species is the initiator of dental caries, patient information on caries risk and oral hygiene practices were not collected. This information may have been beneficial to increase the probability of finding *S. mutans* in samples by targeting high caries-risk patients. Alternatively, targeting patients with poor oral hygiene and no history of dental caries in their lifetime may also have proven significant, since it could be hypothesized these patients may have presence of bacteriophage, which would explain their lack of dental caries even with inadequate oral hygiene.

Another possible explanation for low *S. mutans* percentages may have been due to the time and temperature at which collected samples were preserved. In this study, plaque and saliva samples were stored at -80°C in their respective collection media (AS-920 or vials containing DNA/RNA shield). A study published in 2018 by Cota et el., concluded that samples of *S. mutans* stored at -80°C in brain-heart infusion media (BHI) with 20% glycerol added resulted in a survival rate of 99.7% over a period of approximately 8 years [27]. The study concluded that the addition of a preserving agent, glycerol, was crucial for bacterial cell survival, since it prevents cell dehydration and thus death. Although this study used different storage media, the lack of a preserving agent may have attributed to low bacterial survival rate and thus limited *S. mutans* within samples.

Due to the laboratory results and limited data and analysis of bacteriophage from collected samples, we were unable to reject our null hypothesis that there would be few or no phages against *S. mutans* in clinical plaque and saliva samples.

Since there was lack of phage isolation, we were also unable to reject our second null hypothesis that infection with phage(s) does not prevent or slow the initiation or progression of dental caries.

CONCLUSION

Bacteriophages may be used as a plausible clinical treatment modality for future caries prevention strategies, however discovery and isolation are obstacles that may prevent or hinder the clinical application of phage therapy.

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