Measuring the Relationship between *S. mutans* Levels and Dental Caries Severity in Patients at the MSSU Dental Hygiene Clinic

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In

Biology

A Thesis Presented to the Honors Program at Missouri Southern State University

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Fall 2015

Abstract

Bacteria living in the oral cavity, such as *Streptococcus mutans*, can ferment sugars to produce acids, which can damage teeth. Dental caries (tooth decay) currently affects more than two billion people worldwide. Consensus has not yet been reached regarding how strongly *S. mutans* contributes to dental caries: some researchers reason that *S. mutans* is the primary contributor to this disease, while others claim that various species may be significant contributors. The purpose of this study was to determine if there was a correlation between the *S. mutans* concentration and the plaque index in 24 patients at the Missouri Southern Dental Hygiene Clinic. *S. mutans* DNA was extracted from each dental plaque sample. A quantitative PCR assay was used to quantify levels of *dexA*, a gene coding for dextranase, which breaks down sugars in plaque. The concentration of *dexA* correlated with the concentration of *S. mutans* in each sample. We hypothesized that there would be a strong correlation between the *S. mutans* level and dental caries severity. Linear regression analysis indicated that there was no correlation between the *S. mutans* concentration and plaque index (R^2 = 0.0248). Our results suggest that *S. mutans* does not strongly contribute to dental caries.

Introduction

I chose to conduct research in this topic for various reasons. The MSSU Biology Department acquired the Thermo Fischer Quant Studio 3 Quantitative PCR (qPCR) machine this year. This machine represents cutting-edge technology used by researchers in the fields of genetics and molecular biology. The qPCR process takes only an hour to detect certain genes and pathogens in clinical samples. This is much faster than growing bacteria on petri dishes and waiting for 24 hours to obtain results. I wanted to gain a deeper understanding of qPCR so that I could use this technique during my post-graduate education.

I was also interested in gaining the skills needed to excel in professional or graduate school. Previously, I had conducted experiments in sheltered environments, in which I followed step-by-step, foolproof procedures created by professors and I obtained predictable results. However, in the real world, researchers develop their own procedures and they do not know what results to expect after completing their experiments. I wanted to gain experience with creating my own lab procedure, in which I could determine the gene, chemicals, and temperatures to use in my own experiment. This would allow me to learn how to brainstorm a scientific question and use the resources available in a lab to test this question. I was also excited to further develop my technical, hands-on skills in the lab. I expected this project to expose me to the realities of working in a lab.

I was also curious to learn more about dental caries, which is a highly prevalent disease in the U.S. Having had personal experiences with dental plaque as a child, I wanted to explore this disease further. I knew that bacteria grow in the mouth and form plaque. However, the details of this condition have not been publicized as much as more serious infections like tuberculosis and hepatitis. Which bacterial species are responsible for causing dental caries?

What happens in this disease at the molecular level? Which steps could be taken to improve the effectiveness of treatments used for dental caries? I appreciated the fact that I had the perfect facilities that could be used to explore these questions in depth. Clinical samples could be collected from the MSSU Dental Hygiene Clinic and virulence genes could be studied in the MSSU DNA Lab.

Another reason I chose to do this project was because I had an inherent interest in microbiology and genetics. I enjoyed learning about how microbiological and genetics procedures can be used to make advancements in medicine. For instance, antibiotics can be tested to determine their effects on various bacterial species. I was interested in bridging my interests in dental hygiene, microbiology, and genetics.

Mechanism of Dental Caries

Normal teeth are comprised of dentin, which is covered by enamel. Dentin is a hard tissue that protects the pulp and root canals of each tooth (1, 2). Eating sweets can provide oral bacteria with sugar. Dental caries is caused by bacteria that ferment sugars to produce acids that can dissociate into hydrogen ions. To counteract the increase in hydrogen ions, the teeth release certain minerals, such as phosphate and hydroxyl ions, which react with hydrogen ions to form new compounds. This chemical reaction consumes the reactants, and the level of hydrogen ions decreases (1, 2). At the same time, however, a new problem arises: the reduction of mineral levels forces the teeth to break down hydroxyapatite, which is the foundation of normal teeth. It is degraded to replenish these minerals, thereby destroying the teeth and enamel (1, 2). Severe dental caries can damage the enamel, allowing dentin to get exposed, which can cause pain in the pulp. Over time, bacterial growth leads to an accumulation of plaque on the surface of the tooth.

After a large amount of enamel is lost the tooth surface decays and forms a cavity (3). Overall, dental caries can lead to the breakdown of several major tissues of the teeth.

Measuring Dental Caries Severity

Dental caries severity is determined by using the O'Leary's Plaque Index to measure the level of plaque present in a patient's mouth. Each tooth is checked for plaque and is given a score if it contains plaque. The individual scores are added up, and the total score is divided by the total number of teeth present. Then this quantity is divided by 100 to obtain the percentage of plaque present in the mouth. A "light" rating indicates 1-10% plaque, a "moderate" rating indicates 11-40% plaque, and a "heavy" rating indicates more than 40% plaque.

Fermentation by various bacterial species in the oral cavity greatly contributes to the development of dental caries. Dental caries affects numerous people in the U.S. and all over the world. Dentists generally rate the severity of dental caries by measuring the plaque level in the oral cavity. Fluoride solutions are used to inhibit the growth of whatever bacterial species may be present. Dental professionals physically remove plaque and advise patients to eat fewer sugary products. These treatments generally strive to eliminate as many bacteria as possible, but these treatments do not usually target specific bacterial species. A major cause of this could be that consensus has not yet been reached regarding which bacterial species is/are the biggest contributor(s) to dental caries.

Possible Bacterial Causes of Dental Caries

Some researchers argue that *S. mutans* and closely related species are the major contributors to dental caries. In a 1986 review article, Loesche described the results of various experiments that compared the bacterial and chemical environments of decayed teeth vs. healthy teeth. Acids produced by bacterial fermentation can dissociate into negatively charged particles. These studies concluded that the mutans streptococci, comprised of *S. mutans*, *Streptococcus sobrinus*, *Streptococcus ratti*, *Streptococcus criceti*, *Streptococcus downei*, and *Streptococcus macacae*, can survive at a pH of 5.0, which represents a highly acidic environment (5). Their high intracellular potassium concentrations can neutralize the excessive negative charge. Dental caries involves the production of acids that lead to breakdown of certain tissues in the teeth (2). So various unrelated bacterial species may not cause this condition because they would probably not be able to survive in an acidic environment.

In a 2012 study, Beall, et. al examined how the bacterial compositions of the oral cavity changed as dental caries worsened in participants. Their findings support those described by Loesche. The researchers found that the most common bacterial species found in the oral cavity was usually *S. mutans*. It was also found that as the disease progressed, the levels of *S. mutans* generally increased. This may have occurred because of the acids produced by bacteria. Acid-sensitive species died while acid-tolerant species like *S. mutans* survived. This caused the diversity of the bacteria to decrease (6). Overall, *S. mutans* was the dominant pathogen present in most children with dental caries. The level of this species also grew as the disease progressed.

However, in a 2002 review article, I. Kleinberg proposed that the cause of dental caries is a non-specific mixture of bacteria. Many experiments have found that various species can produce acids that can lead to demineralization of the teeth, so they can also contribute to dental caries. In some experiments, caries lesions still developed when low levels of *S. mutans* and high levels of unrelated species were present (7). So various species may cause dental caries and the species that are responsible may vary from case to case.

Findings from a 1996 study conducted by van Houte, et. al supported the ideas presented by Kleinberg. These researchers grew bacteria in a neutral solution normally found in teeth. Then they added sugar to this solution to mimic severe dental caries. The researchers examined how each species affected the pH of the solution. They found that various bacterial species reduced the pH to amounts less than 4.2, including 100 percent of mutans streptococci and Lactobacillus, 20 percent of the Actinomyces strains, and most of the *Bifidobacterium* strains and non-mutans streptococci (8). So *S. mutans* may not be the only major contributor to dental caries. Numerous bacterial species can make the solution acidic, so they can also cause dental caries.

Several studies have demonstrated that *S. mutans* and other closely related species are the only ones that significantly contribute to dental caries. However, other sources reason that various unrelated species may also significantly contribute to dental caries because they can also produce and tolerate acids in the oral cavity. So the mutans streptococci may not be the only major pathogens involved. It would make sense to assume that if the amount(s) of the primary pathogen(s) increase(s), then the disease should increase in severity. But consensus has not yet been reached regarding the primary pathogen(s) involved in dental caries. So it is not known which bacteria increase in number as the disease progresses.

What if there was a way to take this idea to the next level? Whether or not my results indicate that *S. mutans* is the strongest contributor, my findings can serve as the basis for further studies in people with dental caries. Once consensus is reached regarding the primary pathogen(s), theoretical ideas can be translated into clinical treatments. Dentists could tweak the standard treatments to focus on eliminating the specific pathogen(s) that contribute most strongly to dental caries. Chemicals could be designed to target these bacterial species, and dentists could use these chemicals in dental treatments. Quantitative PCR methods could be used to measure the bacterial levels in patients before and after treatments.

Such methods could increase motivation in patients. In an online article of “Registered Dental Hygienist,” Lisa C. Wadsworth, a dental hygienist, explained that PCR tests could be used to increase a patient’s motivation to maintain proper oral hygiene. It can be difficult for a patient to view damaged teeth or to feel pain in the mouth; these factors can reduce the motivation to take proper care of one’s teeth (9). But if patients could understand how much bacteria is lurking in their mouths, they would be more motivated to brush more often or to eat fewer sugary foods. In general, an understanding of the relationship between bacterial levels and disease severity can be used to improve the quality of dental caries treatments.

Using Quantitative PCR to Measure Bacterial Levels in Plaque

Polymerase chain reaction (PCR) is a process used to make several copies of DNA, or amplicons, from a sample. These amplicons can then be tested for the *presence* of a particular gene. The materials needed for PCR are DNA polymerase, deoxynucleotide triphosphates (dNTP’s), forward and reverse primers, buffers, salts, and template DNA, or the DNA that is tested (10, 11). DNA polymerase uses nucleotides to synthesize DNA strands. The forward and reverse primers bind to the beginning and end of the target sequence. Buffers and salts provide an optimum chemical environment in which DNA polymerase can work efficiently (10, 11). The first step of PCR uses a high temperature to separate the DNA strands. Then the temperature is lowered so that each primer can bind to one of the DNA strands. Lastly, the temperature is raised again so that DNA polymerase can add nucleotides that can bind to each strand to produce complementary DNA strands (10, 11).

Quantitative PCR is a form of PCR that can be used to measure the *amount* of a particular gene in a sample (10, 11). It has recently been used to quantify virulence genes of bacteria contributing to dental caries. These genes are used by organisms to cause diseases. The levels of a virulence gene can be measured in dental plaque samples from people having low, moderate, and severe forms of dental caries. This experiment will measure the level of *dexA,* a gene that allows dextranase to be produced. This enzyme is used to break down sugars in dental plaque. The *dexA* gene is often found in *S. mutans* strain UA159, which can contribute to dental caries. The amount of correlation between the *dexA* concentration and disease severity will indicate how strongly *S. mutans* contributes to dental caries.

Materials and Methods

Tryptic Soy Agar (TSA) plates were used to facilitate the isolation of *S. mutans* colonies by identifying white colonies after 24 hours of incubation at 37 C. Isolated colonies were transferred to additional TSA plates and incubated for 24 hours at 37 C. These plates would be used to prepare the DNA standard.

Preparation of DNA Standard

Overall, DNA was extracted from *S. mutans* found on TSA plates in order to prepare a standard that could be used to generate a standard curve. Later, this graph would be used to determine the concentrations of DNA in patients’ samples. Each bacterial species requires a certain temperature and incubation time to allow for maximum DNA extraction. It was not known which temperature and time were suitable for *S. mutans*, so a time study was conducted to determine these values.

First, bacteria were collected from TSA plates and mixed in a vial containing Quick Extract ^{TM} DNA Extraction Solution (Epicentre Biotechnologies, U.S.A.). *S. mutans* was incubated at 37 C and was removed from the incubator every 15 minutes. Bacterial samples that had been incubated for 0, 15, 30, 45, and 60 minutes were spun and centrifuged to form solutions containing a supernatant (DNA liquid) and a bacterial pellet. The concentrations of the supernatants were measured. It was found that the sample that was incubated for 30 minutes had the highest DNA concentration. Then the previous steps were repeated, except that *S. mutans* was incubated for 30 minutes. The DNA was extracted, and the concentration of the DNA standard was found to be 6.5 ng/uL.

Generation of Standard Curve

Specific sequences of the primers were used to amplify selected portions of the *dexA* gene (Table 1). To determine the concentration range of *S. mutans* DNA, a 10-fold dilution series of the DNA standard was prepared from 6.5 to 6.5 x 10^{-6} ng/uL. Ten uL of each solution was added to 2 MicroAmp® Optical 8-Tube Strips. Each tube also received 15 uL of a mixture containing 5 uL of 100 um of forward and reverse primers, 250 uL of a mixture of DNA polymerase and dNTP’s, and 40 uL of DNA-free water. Additional DNA-free water was used to adjust the reaction mix to a final volume of 25 uL. The reaction was done in duplicates. Amplification and detection of the *dexA* gene were done using the QuantStudio ™ 3 Real-Time PCR System with the following cycle profile: Initial denaturation at 95 C for 5 minutes, followed by 40 cycles of amplification at 95 C for 15 seconds and 60 C for 30 seconds. Then a melting curve analysis was conducted using the following cycling parameters: 95 C for 15 seconds and

60 C for one minute. Lastly, the temperature was slowly increased to 95 C. A standard curve was generated.

Designation	Sequence (5’-3’)	Amplicon size (bp)	Target gene
Primers			
Mutans reverse	5’-GCTCCACCAATTCCAAGACT-3'	231	*dexA*
Mutans forward	5’-GCTATTGCGGTTGAGGATGA-3'	231	*dexA*

Table 1. Oligonucleotides used for qPCR amplification of *S. mutans*. The information about primers was found in an article covering an experiment conducted by Goto, et. al in 1996.

qPCR Matrix Spiking

Matrix spiking involves measuring the ability of a procedure to provide accurate and valid results (13). One sample is “spiked” with a bacterial gene while the other sample lacks this gene. Here, the gene concentration of the “spiked” sample is unknown. The goal is to ensure that the procedure can work effectively with one bacterial sample before it is used on experimental samples. A sample from the researcher’s teeth was obtained and mixed with 100 uL of the previously mentioned DNA extraction solution. The solution was incubated at 37 C for 30 minutes, then spun and centrifuged again to form a bacterial pellet and supernatant. Sixty uL of the supernatant was collected and divided equally into two vials. One vial received 20 uL of water while the other received 20 uL of the DNA standard. These were the sample + water and the sample + standard (spike) solutions. Then a 10-fold dilution series was prepared from each mixture. Each mixture received DNA-free water, DNA polymerase, dNTP’s, and primers that were used in the previous experiment. The same times and temperatures used for amplification and quantification in the above experiment were used here.

Since the spike sample experiment represents a quality control test, it is supposed to provide certain results. The presence of these results indicates that the procedure worked effectively. The only difference between the sample + standard solution and the sample + water solution is whether or not the gene is present. So the Ct values of the first solution should be overall smaller than those of the second solution.

In addition to these mixtures, a mixture of standard + water was tested. It contained known concentrations of the standard so that it could serve as a positive control (it should have *dexA*). For this reason, this mixture was hypothesized to contain the highest levels of *dexA* and therefore have the lowest Ct values. Each of the three mixtures was added to eight wells of a strip labeled A-H (Fig. 1). The first mixture was added to wells A6-H6, the second mixture was added to wells A7-H7, and the third mixture was added to wells A8-H8. In each strip, wells labeled “A” had $6.5x10^{-1}$ ng/ul of a mixture. Each subsequent well contained a concentration that was 10 times smaller. Each well labeled G contained $6.5x10^{-7}$ ng/ul. Each well labeled H contained only water.

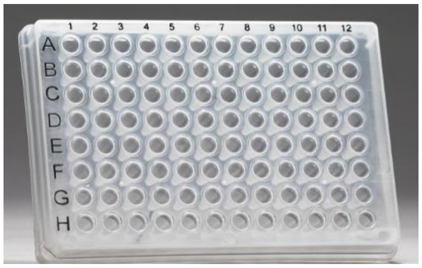

Figure 1. An example of a 96-well plate used for qPCR assays (14).

Collection of Patient Samples

Plaque samples from twenty four patients at the MSSU Dental Hygiene Clinic were collected by dental hygienists and given to the researcher. Each sample was designated as having a low, medium, or heavy plaque index. Each patient was identified based on an assigned number. Eight samples of water would comprise the negative control group, since these lacked *dexA*.

Next, the DNA was extracted from each sample using the previously mentioned steps. Ten uL of each sample's DNA extract was added to a tube in the MicroAmp® Optical 8-Tube Strips. Then 90 uL of water was added to each tube so that each sample could be diluted 1:10. Each sample received 15 uL of a mixture containing 9 uL of forward and reverse primers, 72 uL of DNA-free water, and 450 uL of DNA polymerase and dNTP's. SYBR Green I dye (Thermo Fischer, U.S.A.) was added to each sample. Four strips of eight wells were tested and each contained one of the following groups: DNA standard, samples from patients 1-8, samples from patients 9-16, and samples from patients 17-24. Four wells contained water and served as the negative control. Amplification and quantification of the *dexA* gene was achieved by using the same temperatures and times used in the above experiments. Lastly, 5 uL of each sample's DNA extract was added to a spectrophotometer so that the concentration of a general form of DNA (common to all living organisms) could be measured. This was done to verify that each sample contained some form of DNA.

Quantitative PCR Terminology

In general, a qPCR assay uses DNA polymerase, dNTP's, forward and reverse primers, and a fluorescent dye. DNA polymerase joins dNTP's together to form the DNA strands. The amount of fluorescence produced by this dye correlates with the gene's concentration and can be used to measure this level. During the beginning of the reaction, changing reaction conditions cause the amplicon levels to fluctuate, so the fluorescence also keeps fluctuating (15, 16). The machine creates a background signal that is generated and is used to calculate the baseline fluorescence. When a significantly large amount of amplicon has been produced, a signal called the threshold is generated that is significantly greater than the baseline signal (15, 16).

The amplification plot represents the amount of amplicon produced throughout the reaction (15, 16). This plot is analyzed to determine the number of DNA replication cycles completed when a sample produces enough amplicon that its signal crosses the threshold. This occurs during the exponential phase of the reaction when the highest amounts of reagents are available, so the highest amount of DNA is produced. The graph compares the number of cycles completed when the fluorescence produces a signal that is just above threshold (15, 16). The greater the amount of gene initially present in a sample, the fewer cycles would be needed to reach threshold. So samples containing greater levels of a gene would have a smaller cycle value.

The standard curve is used to compare the logarithm of the gene concentration with the number of cycles needed to reach threshold. It involves using a series of diluted samples with known concentrations. The range of these concentrations should be wide enough to include expected concentrations of the experimental samples (15, 16, 17). After the C_t values of the experimental samples are determined, the standard curve can be used to interpolate the gene concentrations of these samples. The standard curve can also be used to evaluate the accuracy of the reaction.

Results

Initially, a standard curve was obtained by amplifying *dexA* found in the *S. mutans* DNA standard (Fig. 2). It was found that samples with higher *dexA* concentrations required fewer cycles of replication to obtain the threshold signal than those that were more diluted. The C_t value represents the number of cycles needed to reach threshold. Samples containing greater amounts of *dexA* had smaller Ct values. The R^2 value showed that there was a strong correlation between the DNA concentration and Ct value. Each concentration was obtained by using the following equation: $0.65 * (10^{-\text{dilution factor}})$. This equation was also used to calculate the concentrations for the spike sample experiment.

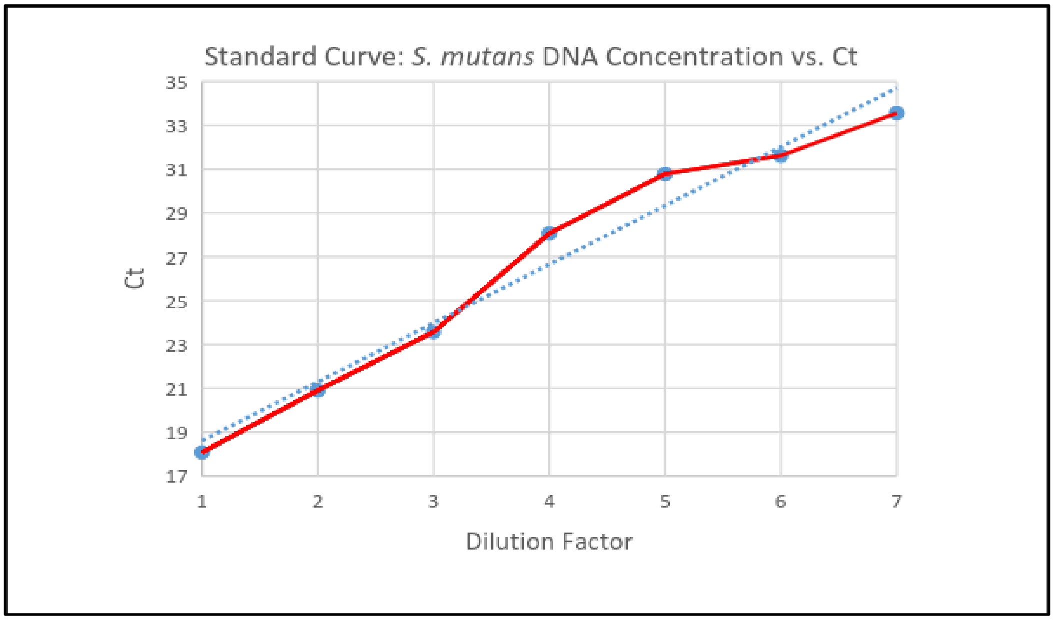

Figure 2. Standard curve of the qPCR assay obtained by the amplification of *dexA* using 10-fold dilutions of bacterial DNA standard. The R^2 value was 0.9703.

A graph was generated that compared the C_t values of the spike sample (Fig. 3) with the one containing sample and water (Fig. 4). Both solutions were diluted to concentrations ranging from 6.5×10^{-1} ng/uL to 6.5×10^{-6} ng/uL. The dilution factor ranged from 1-7. Both graphs showed that as the dilution factor increased (concentration decreased), the C_t value increased. The R^2 values of these curves showed a strong correlation between the DNA concentration and Ct value. So a lower concentration of *dexA* required a greater number of cycles to reach threshold. The spike sample had a greater amount of *dexA*, so it had lower C_t values.

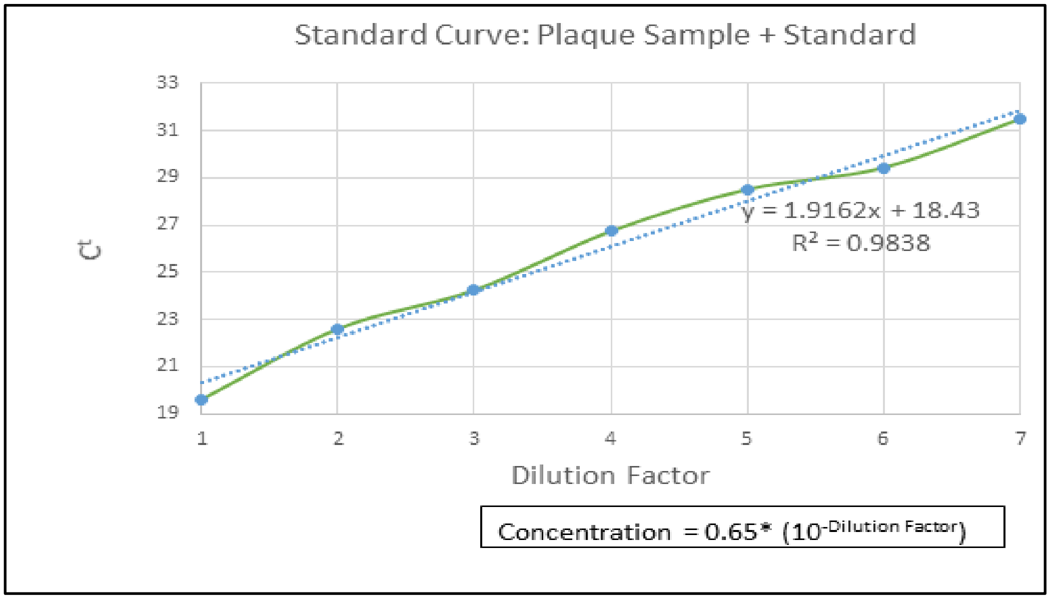

Figure 3. Ct vs. concentration curve obtained by amplification of *dexA* using 10-fold dilutions of a bacterial spike sample. The R^2 value was 0.9838.

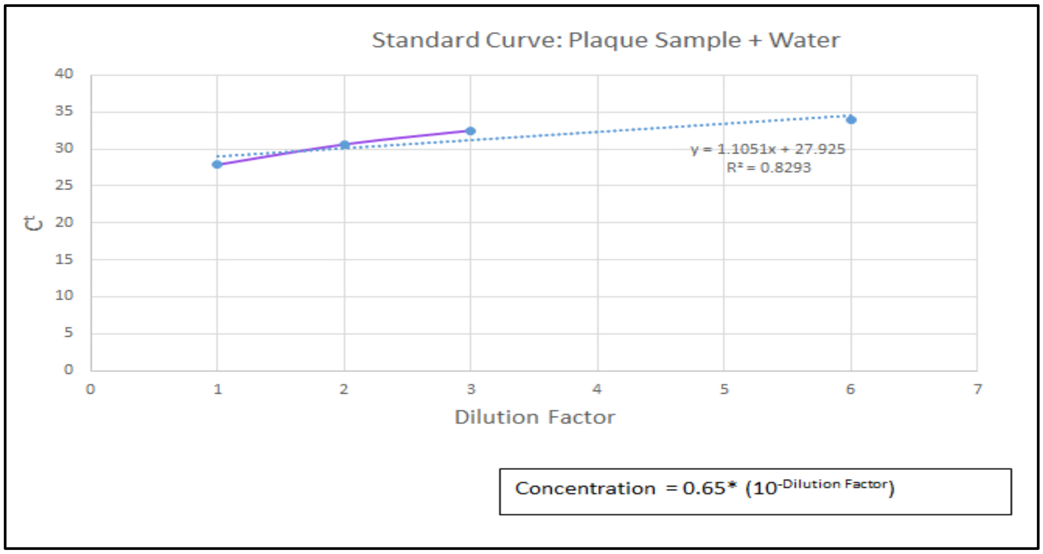

Figure 4. Ct vs. concentration curve obtained by amplification of *dexA* using 10-fold dilutions of a sample containing plaque and water. The R^2 value was 0.8293.

An amplification plot was also generated by amplifying *dexA* in the DNA standard (Fig. 5). The threshold is represented by locating the point of intersection of the amount of signal with the number of cycles needed to reach threshold. The threshold is found in an area where each sample's curve increases linearly. This graph shows each sample's number of cycles needed to reach threshold. The samples represented by the curves on the right had smaller levels of *dexA*.

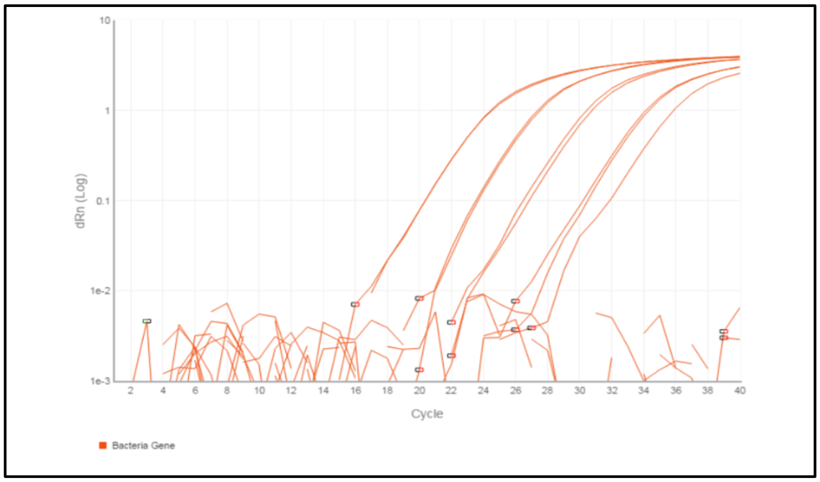

Figure 5. Amplification plot of the qPCR assay obtained by amplification of *dexA* using 10-fold dilutions of bacterial DNA standard.

An amplification plot was generated that compared the signal of a sample containing plaque and the DNA standard ("spike sample") with one containing plaque and water (Fig. 6). The sample containing the standard required fewer cycles to reach threshold than the one containing water.

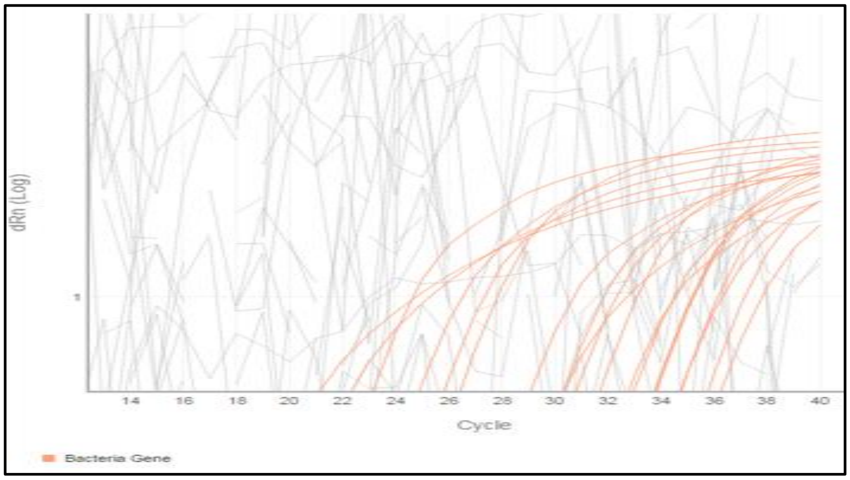

Figure 6. Amplification plot of the qPCR assay obtained by amplification of *dexA* using 10-fold dilutions of a bacterial spike sample.

Plaque found in the posterior molars was collected from 24 patients and *dexA* from each sample was amplified. A table with the patients' numbers, Ct values, *dexA* concentrations, and plaque indices (Table 2) was generated. In addition, an amplification plot (Fig. 7), a gene concentration vs. plaque index curve (Fig. 8), a gene concentration vs. dental caries severity graph (Fig. 9), and a DNA concentration vs. plaque index curve (Fig. 10) were generated.

qPCR Results of Patients' Samples				
Patient	**Ct**	**Dilution Factor**	**Concentration of *dexA* (ng/uL)**	**Plaque Index**
1	36.875	7.81	1.00673E-08	54%
2	31.803	5.92	7.81472E-07	35%
3	33.088	6.4	2.5877E-07	35%
4	32.376	6.13	4.81852E-07	25%
5	40.000	8.98	6.80634E-10	42%
6	27.902	4.46	2.25379E-05	80%
7	24.575	3.22	0.000391664	68%
8	30.254	5.34	2.97107E-06	87%
9	40.000	8.98	6.80634E-10	60%
10	34.900	7.08	5.40646E-08	42%
11	40.000	8.98	6.80634E-10	67%
12	35.206	7.19	4.19675E-08	70%
13	31.826	5.93	7.63683E-07	20%
14	40.000	8.98	6.80634E-10	67%
15	34.997	7.11	5.04561E-08	52%
16	33.957	6.72	1.23855E-07	4%
17	27.761	4.41	2.52879E-05	25%
18	40.000	8.98	6.80634E-10	35%
19	40.000	8.98	6.80634E-10	67%
20	40.000	8.98	6.80634E-10	20%
21	34.674	6.99	6.6514E-08	5%
22	27.445	4.29	3.3336E-05	75%
23	40.000	8.98	6.80634E-10	91%
24	40.000	8.98	6.80634E-10	92%

Table 2. Ct values, dilution factor, gene concentration, and plaque index of patients' samples obtained by amplification of *dexA* using 1:10 dilutions of patients' plaque samples.

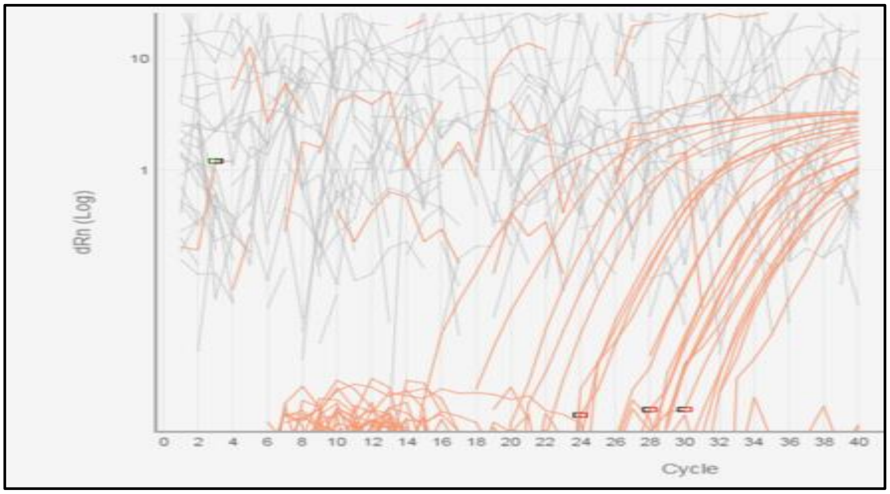

Figure 7. Amplification plot of the qPCR assay obtained by amplification of *dexA* using 1:10 dilutions of patients' plaque samples.

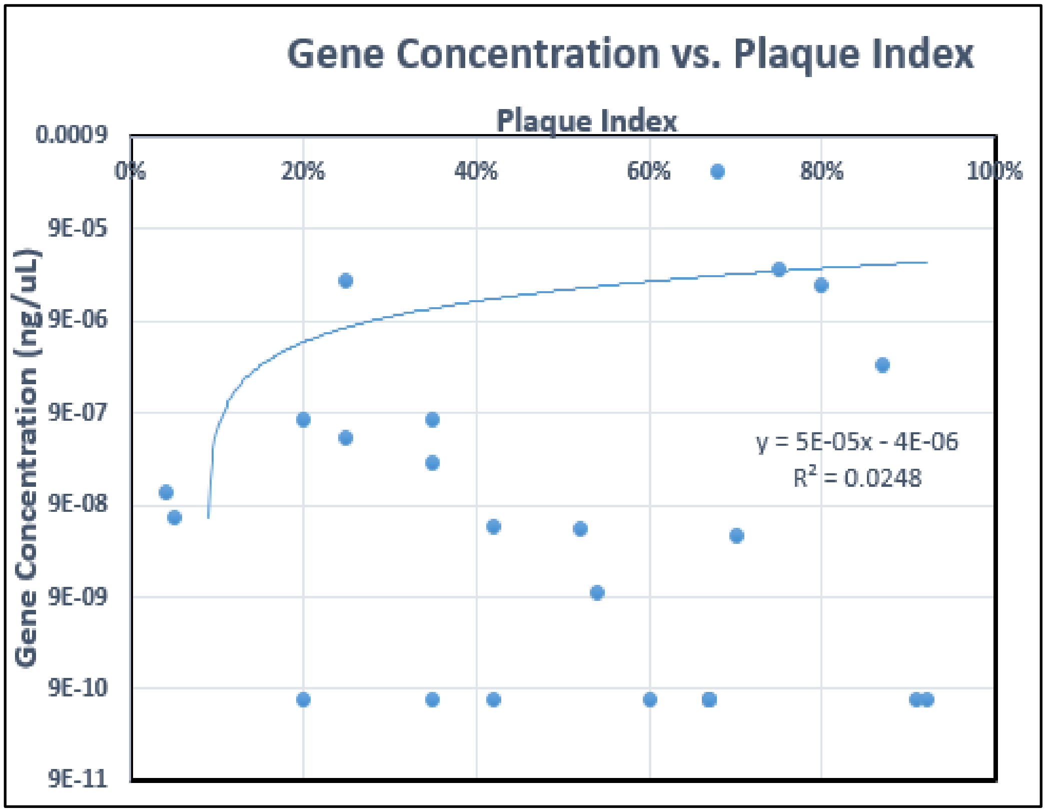

Figure 8. Gene concentration vs. plaque index curve obtained by amplification of *dexA* using 1:10 dilutions of patients' plaque samples. The R^2 value was 0.0248.

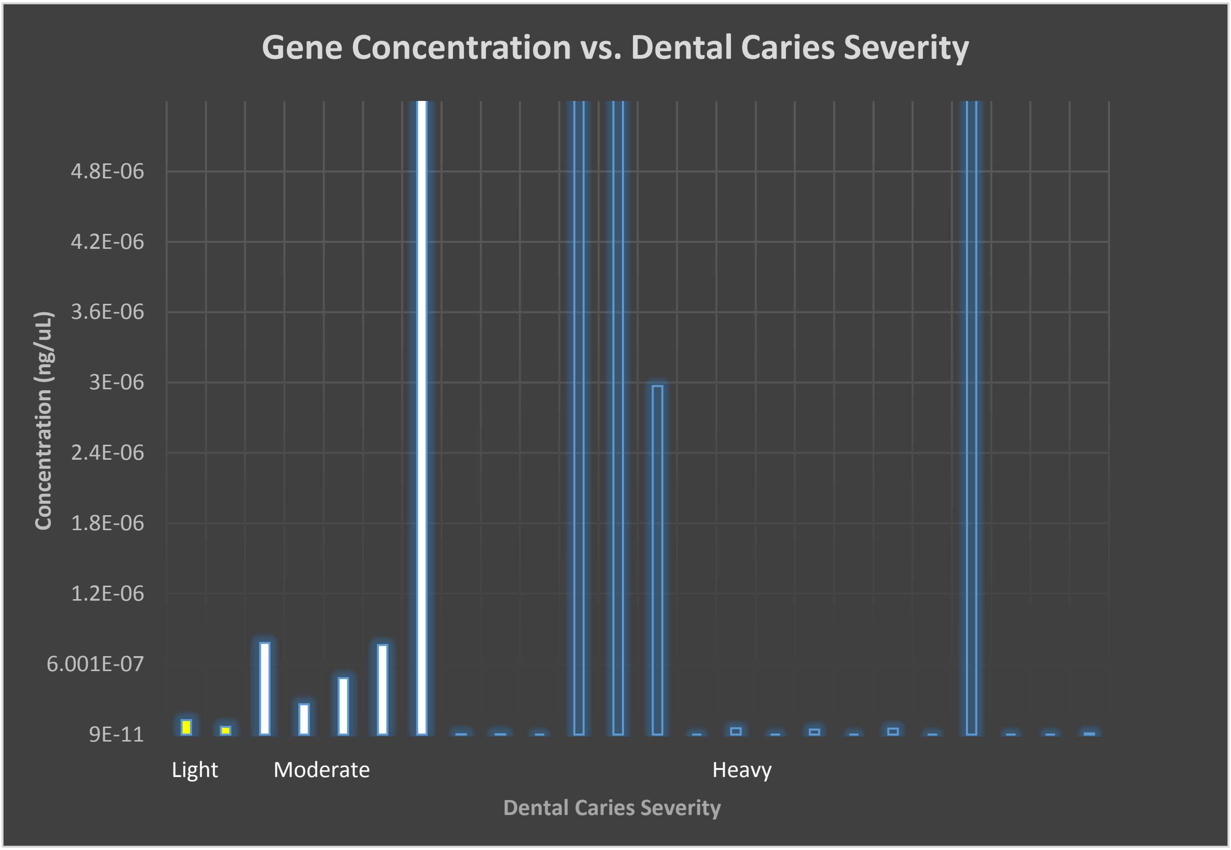

Figure 9. Gene concentrations for each level of dental caries severity. There was a wide range of gene concentrations for each category.

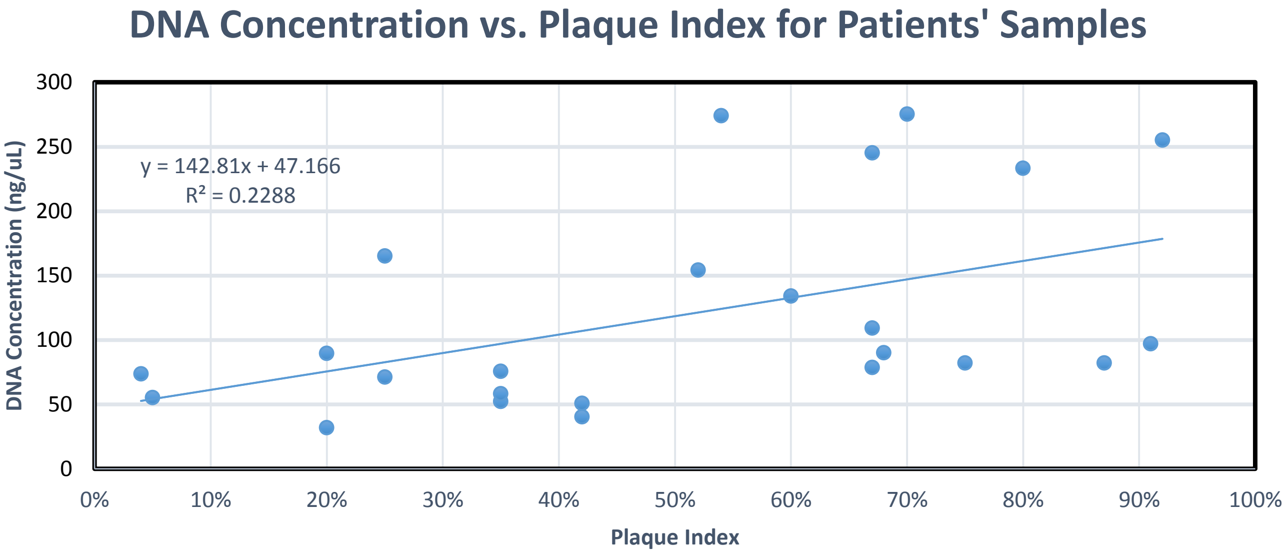

Figure 10. DNA concentration vs. plaque index curve for patients' samples. The R^2 value was 0.2288.

Discussion

Standard Curve and Spike Sample Experiments

First, it was found that each plaque sample contained some form of DNA (Fig. 10). This test was used to check if each sample even contained any DNA at all. If a sample did not contain any DNA, then the possibility of it having *dexA* could be ruled out. It was found that all of the samples contained relatively high levels of DNA, so there was a chance that they all had *dexA*. There was no correlation between the plaque index of a patient's sample and the DNA concentration.

For the standard curve experiment, it was found that there was a strong linear correlation between the dilution factor and Ct value. This is expected since lower concentrations are more diluted and should contain smaller levels of *dexA*. Smaller gene concentrations require a greater number of cycles to reach threshold. The strong linear correlation indicates that the standard curve is valid and can be used to accurately obtain the gene concentration of each sample.

For the sample + standard, and sample + water curves, there was a strong linear correlation between the dilution factor and Ct value (Figs. 2-4). This makes sense since a greater dilution factor indicates a lower amplicon concentration. Therefore, a greater number of replication cycles will be needed to reach threshold. Both experiments had melting curves with one peak, so only *S. mutans* DNA was present. The samples that were tested were therefore pure. If primer-dimers or DNA from other organisms were present, then more than one peak would have been generated. A primer-dimer is a structure made when complementary bases on both of the primers attach to each other. This is not supposed to form during the reaction. Otherwise, this process interferes with the formation of the PCR product, so amplification does not work as well, which causes less product to be formed.

For the spike sample experiment, it was found that diluted concentrations of the spiked sample contained significantly high levels of *dexA*. This can be understood by comparing the Ct values for the same concentrations of the sample + water and sample + standard solutions. At a concentration of 0.65 ng/uL (dilution factor of 1), the Ct value for the sample + water solution was 27, but the Ct value for the sample + standard solution was 19. The sample + standard solution had a lower Ct value, since it contained more *dexA* than the other sample. This makes sense since the standard is the positive control and is therefore supposed to contain a high level of *dexA*. The spike sample's gene concentration was significantly higher than that of the negative control (sample + water). The researcher's sample was not expected to contain any *dexA*. The results obtained from the spike sample experiment validated our findings.

Data from our population's samples does not support our hypothesis of a positive correlation existing between the plaque index and *dexA* concentration. Since *dexA* is found in *S. mutans*, this gene's concentration should be the same as the bacterial concentration. The R^2 value was 0.0248, indicating a weak correlation between the *S. mutans* concentration and dental caries severity.

Which factors could have contributed to these findings? As previously mentioned, no consensus has been reached regarding which bacterial species is the most significant contributor to dental caries. Some sources reason that dental caries can be linked to *S. mutans*, while others argue that dental caries is linked to several species. My findings more closely match those found by the latter group of researchers.

First, some scientists reason that *S. mutans* and some of its relatives play a major role in causing dental caries. *S. mutans* breaks down a variety of sugars to generate acids and lower the pH of the oral cavity. How does *S. mutans* protect itself when the pH becomes too acidic? It uses an acid tolerance mechanism: it first exposes itself to a pH of 5.5 (moderately acidic), which triggers the expression of genes needed for survival at a lower (highly acidic) pH (18, 19, 20). These genes control processes that involve repairing cellular components such as membranes and DNA that are often damaged by acids. These processes also involve shifting the pH back to around 7 (normal level) by increasing potassium concentrations inside the cell. This helps to neutralize the excessive negative charge in the cell that was generated by acids.

In contrast, several species competing with *S. mutans* in the oral cavity lack some of these sophisticated protective mechanisms, so they often die in highly acidic environments (18, 19, 20). In a 2000 study conducted by Faustoferri, et. al, it was found that *S. mutans* adapted more effectively to a pH of 5 than *S. sobrinus*. *S. mutans* did this by modifying the structure of its cell membrane to include more unsaturated fats in order to block the passage of hydrogen ions into the cell. If hydrogen ions enter a cell, they can make the cell more acidic and destroy cellular components. The new membrane prevented the cell from becoming more acidic, which allowed *S. mutans* to adapt to the acidic environment. *S. sobrinus* did not modify the structure of its membrane, however, so it was not able to adapt as well to a pH of 5.

Another reason why *S. mutans* may be the primary contributor to dental caries could be because it may destroy other bacteria in the oral cavity. This can allow *S. mutans* to obtain more nutrients, such as sugar, in the oral cavity so it can achieve lots of growth. In review articles by Merritt and Qi and Kreth, et. al, the researchers explained that *S. mutans* can produce mutacins, which are chemicals that can inhibit the growth of numerous bacterial species in the oral cavity. Mutacins I and IV are secreted to inhibit the growth of *Streptococcus sanguinis*. In a 2000 study, Caufield, et. al examined the role of *S. sanguinis* in infants for three years. They found that this species serves as a commensal microbe in the oral cavity. Such microbes naturally live in the body and generally provide nutrients or protect the body from pathogens. In this case, *S. sanguinis* tries to obstruct efforts made by *S. mutans* to cause dental caries. Children having low levels of *S. mutans* had significantly higher levels of *S. sanguinis*. In a 2015 study, F. Teng, et. al found that several groups of children with varying severities of dental caries had similar levels of *S. mutans*. These findings show that *S. mutans* tries to get rid of bacterial species that come in the way of its growth. It is possible for *S. mutans* to use the above tactics to dominate as the champion of the "survival of the fittest" game in the oral cavity. For these same reasons, *S. mutans* may contribute strongly to dental caries.

On the other hand, some researchers reason that dental caries should be viewed as a "multispecies" disease. In a 2004 study conducted by Abranches, it was found that *S. sobrinus* also uses various mechanisms to cause dental caries. It uses the sugar phosphotransferase system to transport sugar into the cell to produce energy at low pH levels. So even in an acidic environment, *S. sobrinus* can continue to transport sugar and maintain adequate energy in the cell. This allows it to survive in highly acidic environments, which makes it more likely that it can cause dental caries. Of course, *acid production* by itself is not enough to cause this disease. If a species has strategies for *surviving* in an acidic environment, then it can achieve long-term growth in the mouth. Then over time, bacterial communities can grow on the teeth and cause dental caries. Since *S. sobrinus* can sustain growth in an acidic environment, it could also contribute to dental caries.

A 2015 study conducted by Hayashi, et. al also concluded that *S. sobrinus* can strongly contribute to dental caries. The researchers studied the bacterial compositions of three groups of schoolchildren: those with decayed primary teeth, decayed permanent teeth, and decayed primary and permanent teeth. They found that each of these groups had a higher level of *S.*

mutans and *S. sobrinus* combined than *S. mutans* alone. This study shows that *S. sobrinus* may also strongly contribute to dental caries. While *S. mutans* may contribute to dental caries, it may not be the *primary* contributor to this disease.

In a 2002 review article, I. Kleinberg explained that some experiments found that dental caries still developed in subjects having relatively low levels of *S. mutans*. In 1994 and 1996 studies, Van Houte, et. al found that several species other than *S. mutans* produced enough acid in the mouth to be cariogenic (likely to cause dental caries). These species included *S. mitis*, *S. milleri*, and bacteria belonging to the genus *Bifidobacterium*. These findings show that when investigating the causes of dental caries, various species should be taken into consideration.

The findings supporting a multispecies model of dental caries include ideas that may also apply to my experiment. I did not find a strong correlation between the *S. mutans* concentration and dental caries severity. It is possible that in some of the patients, *S. sobrinus* and/or other species contributed more strongly to dental caries than did *S. mutans*. Species such as *S. sobrinus*, *S. mitis*, and *S. milleri* may have produced enough acid to create plaque on the teeth. It could also be possible that strains of *S. mutans* other than UA159 were present in the samples. Of course, this study did not use primers to look for genes unique to other *S. mutans* strains or other bacterial species, so these ideas are only possibilities. Lastly, other genes in *S. mutans* UA159 may have contributed to dental caries, but they were not detected by our experiments.

It was interesting to examine the range of gene concentrations found for each plaque index. Out of 24 patients, 15 had heavy plaque (Table 2). It was interesting to note that in this group, the *dexA* concentrations ranged from 10^{-10} ng/uL to 10^{-4} ng/uL, showing a difference of 10^{6} (one million) ng/uL from person to person. The reason for such a huge difference could be that each person's oral cavity has a unique bacterial composition. Some may naturally have more

S. mutans in their mouths, while some may have more *S. mitis* or *S. milleri*. So it is possible that the concentrations of species other than *S. mutans* strongly correlated with the dental caries severity in some of the patients.

It is also important to consider factors unique to the patients that could have affected the results. Perhaps the patients' lifestyles could have affected the results: the sugar levels of the patients could have affected the *S. mutans* concentrations. It was not possible for the patients' diets to be controlled.

There was a wide variety of plaque indices found in the population. There were 15 patients with high plaque levels, seven patients with medium plaque levels, and two patients with low plaque levels. This allowed us to understand what happened to the gene concentration as the plaque index increased in severity. However, using a relatively small population does not allow one to obtain a highly accurate sample standard deviation or population mean (29). Further studies should measure gene concentrations of a larger number of patients to obtain a more accurate standard deviation. In such a study, the sample mean could be used to precisely determine the population mean (the mean for patients in Joplin).

Reflections

This project taught me various skills that will be useful in medical or graduate school. First of all, I enhanced my technical skills in the lab and got more hands on experience with performing key procedures in biology. I learned how to add chemicals to wells more efficiently. Pipetting is the process of ejecting fluids into wells: it involves turning a dial to indicate the volume that needs to be ejected, then pushing down to expel the fluid into the well. I practiced this skill in my molecular biology class; however, there were tips that could be used to make the pipetting faster and easier that I did not know about.

I learned that holding the pipet at a 45° angle will allow all of the fluid to enter the bottom of a well. Otherwise, droplets can get stuck on the side and not make it to the bottom of a well. In order for the chemicals to get mixed effectively in the well, all of the substances must be at the base of a well. If a few droplets get lost, this changes the volume used in the chemical reaction. Another technique that can affect the reaction volume is forceful pipetting. Pushing on the pipet with too much force can suck the fluid back into the pipet tip, so less volume is actually expelled. One would think that a few droplets should not make much of a difference. I fell into this trap at the beginning of my experiment. After looking at my first results, however, I was shocked.

Biology experiments often involve using volumes that are on the scale of 10^{-6} L. Extremely small volumes are used that are hard to estimate with the naked eye. So it is difficult to look at two fluid samples and tell the difference in their volumes. Both volumes will look like they are equal. So after one has completed pipetting fluid, it is difficult to look at a well and tell if the correct amount of fluid has been added. This is why when a few droplets are lost, it may not look like much has been lost. In reality, however, they could indicate that several uL of fluid have been lost. For example, instead of 20 uL of fluid, one may have pipetted 15 uL of fluid. Overall, I learned that a few droplets can make the difference between accurate results and erroneous results.

Another skill that I developed was the ability to work under pressure and to think creatively to solve problems. I met with my mentor to learn how to use the qPCR Cloud software and qPCR desktop software, as well as how to use the qPCR machine to begin each experiment.

Both of the qPCR software programs were new to my mentor and me, so we both read the user guides and tried out various functions to learn about how they worked. First, the Cloud software is used to view the PCR process in real-time. The software indicates the amount of time remaining until a reaction is completed. I viewed the results of each reaction on this software. The Design and Analysis desktop software allowed me to create the reaction's template. In this program I entered the temperature and duration for each stage of the reaction, as well as the gene that needed to be detected. For each experiment, the machine follows the instructions given by the experiment's template. At first, the idea of creating a template seemed daunting.

However, after meeting with my mentor and after using some trial and error, I learned how to create effective templates. For the beginning experiments, I created templates that instructed the machine to analyze only wells in which chemicals were added. For example, in a 96 well plate, only 24 wells may be used. So the template instructs the machine to look for *dexA* in 24 wells. However, for some reason, the graph created by the machine is not as accurate. My mentor and I did not know this before, so this was a learning experience for the both of us. For the subsequent experiments, we created templates that instructed the machine to look for the gene in all 96 wells. All of these experiences taught me the importance of not giving up in the face of problems. Instead, one should look for alternate strategies and try each one to see which one works.

I also learned how to use the qPCR touchscreen machine to open files, to run experiments, and to transfer files. These are seemingly simple, yet crucial tasks. If the wrong buttons get clicked, one cannot access the results, and an entire qPCR run can get wasted. Keeping this in mind, I worked hard to learn as much as possible about the machine before beginning my experiments. In August, I attended a presentation given by a Thermo Fischer representative at MSSU, during which he provided tips for using the machine efficiently. He explained how to troubleshoot problems with the machine in order to minimize the chances of obtaining erroneous, misleading results. The representative also explained how to run an experiment with empty wells to verify that the machine is working properly. This ensures that when the real experiment is run, it will have the correct independent variables, which will maximize the accuracy of the results (30). The following questions need to be considered when looking at the results of this experiment: Did the template contain the correct temperatures and durations for each interval of the qPCR process? Was the machine instructed to analyze the correct volume of fluid in each well? (31, 32). I followed these steps and ran a blank experiment before running my actual experiments.

These experiences showed me that the actual steps needed to use the machine are actually not very difficult. At the same time, it was challenging to learn and to become comfortable with using unfamiliar procedures in a fast-paced environment. I had to learn how to interpret data from the same experiment that was found on the desktop and Cloud software, as well as the actual touchscreen machine. Over time, however, I became better at using these programs to analyze clinical samples.

This experience also deepened my understanding of the molecular mechanisms, pathogens, and treatments involved with dental caries. Unlike most infections that are caused by one pathogen, such as strep throat, dental caries is a complex condition that can be caused by multiple pathogens. It was interesting to learn that this infection is generally not treated with antibiotics, since less expensive options, like mouthwashes and toothpastes, can be used to achieve the same effects. I became more knowledgeable about the mechanisms used by mere one-celled organisms to wreak havoc in the oral cavities of humans. Bacterial species contributing to dental caries often use sugars and proteins to construct biofilms (33). In these shelters, bacteria can protect themselves from the world of “dangerous” toothpastes and mouthwashes. These seemingly simple species are quite smart, after all.

Completing my honors thesis taught me how to explain a topic by considering multiple perspectives. Not only did I learn how to express my ideas scientifically, but I also learned how to simplify my ideas to make them understandable to the general population. In scientific articles, writers generally use precise, non-figurative language (34). This is because it is easier to understand a specific word, such as “gene” instead of an ambiguous phrase such as “segment of DNA.” The word “gene” only refers to a chemical that has instructions for creating proteins or RNA molecules. In contrast, the phrase “segment of DNA” could refer to genes or to various other chemicals, so the reader cannot understand the intended meaning. Scientific language does not involve imaginative descriptions or figures of speech since these can possibly contain multiple meanings.

This project showed me how to make complex scientific concepts understandable to non-specialists. When writing essays, writers must consider their audiences. For a scientist, the audience is generally other researchers in the same field. At the same time, it is also important to make scientific ideas relevant to a layperson audience. In a 2013 article, Brownell, et. al explained the benefits of pursuing this goal. Having an understanding of basic scientific concepts will allow people to make more informed decisions. For example, I defined various terms before explaining the details of dental caries and qPCR to make these processes easier to understand. I also explained how my findings could benefit members of the general population: genetic research is advancing rapidly and will hopefully translate scientific findings into improved treatments. Using these strategies allowed me to get out of the scientific bubble and to look at the big picture. The purpose of conducting research is to ultimately share new knowledge that can benefit society. Scientists who take the initiative to share their knowledge with the public are more likely to make a lasting impact in the world around them, than those who choose not to do so.

It was interesting to compare qPCR with previous technologies used to study dental caries. During the 19th and most of the 20th centuries, researchers examined the pathogens responsible for dental caries by growing samples on agar in petri dishes and counting the number of bacterial colonies that formed on them (a process known as bacterial cultivation). The higher the number of colonies that was present, the greater amount of bacteria that were present in a sample. The PCR procedure was developed during the 1980's (36). Scientists used this process to detect the presence of virulent genes in clinical samples. Many scientists during these time periods used these procedures to learn about which bacterial species contributed most strongly to dental caries. From the results of experiments that used these procedures, the majority of scientists concluded that *S. mutans* was the most significant contributor to dental caries.

Though these procedures were advanced for their time, today they are less advanced than qPCR. So it is necessary to reconsider the results of past studies in the light of new technology. First, the bacterial level obtained by counting colonies may sometimes be lower than the actual value. Agar plates only show bacteria that are actively growing. There could be dormant bacteria that may not be seen on the plate (37, 38). However, the qPCR machine just measures a gene's concentration. Since it is not looking for bacterial colonies, it is less likely to ignore any bacteria.

Another problem is that agar plates only contain colonies of bacterial species that are able to grow on them. There could be bacterial species found in the sample that are not able to grow on a particular type of agar. In a 2013 review article, Beighton, et. al explained that most of the bacteria that live in the body cannot be grown on agar in the lab. It is possible that some of the bacteria contributing to dental caries may be hard to grow on standard agar. So cultivating bacteria on agar may cause some of the potential pathogens to go undetected. In contrast, qPCR can be used to measure the concentrations of bacteria that are hard to grow in the lab; since it does not look for bacterial growth, it is less likely to ignore these bacterial species. On the whole, qPCR has been shown to provide more accurate results than bacterial cultivation.

The negative part about the PCR procedure is that it cannot measure the bacterial concentration during the PCR process, which qPCR is capable of achieving (39, 40). Detecting the presence of a virulent gene only shows whether or not a bacterial species contributes to a disease. However, qPCR can be used to measure how strongly a bacterial species contributes to an infection.

For these reasons, it is important to reexamine potential contributors to dental caries. Numerous experiments using qPCR have shown that various bacterial species can significantly contribute to dental caries (7, 26, 27, 28). My access to newer technology may allow me to develop a better understanding of the conflicting results of these studies. Over time, technology keeps getting improved, producing instruments that have fewer errors than the previous ones. This can aid with resolving inconsistencies and can open the doors to the next level of research with even more refined instruments.

If I did this project again, I would approach it with a more open mind. This was only my second research project. As a relatively new researcher, I thought that experimental errors indicated problems with my lab techniques. However, my mentor explained that results which look like errors on the surface may actually indicate new knowledge. In science, people may discover new ideas by obtaining results that do not fit the trend set by results from previous experiments. For instance, I found that some of the patients with low plaque levels had higher amounts of *dexA* than some of those with higher plaque levels. I was alarmed at my findings and I discussed them with my mentor. He advised me to consider my results in an unbiased manner.

The problem was that I tried to make my results fit with my hypothesis. I thought that if my results did not match my hypothesis, then the results were erroneous. If *S. mutans* contributes to dental plaque, why doesn't its concentrations increase linearly with the plaque severity? It depends on how *strongly* these variables correlate with each other. Each person's mouth has a unique bacterial composition. The common trend found with *S. mutans* may not necessarily apply to each population studied by researchers. I learned that research involves delving into unknown territory and being unafraid of what the results will indicate. This has been the key to success in science. This represents a stark contrast from the type of thinking encouraged by experiments in classes, in which each student is expected to obtain a similar result. This project has definitely helped me mature as a student researcher.

This project could be expanded in the future to include other genes or other bacterial species or strains of *S. mutans*. Future students can measure the quantities of genes unique to other bacterial species and strains of *S. mutans* to determine if these bacteria contribute to dental caries in patients at any dental clinic in this community. In one project, a student can measure the concentrations of several genes of numerous species! Students can also use qPCR to measure the concentrations of virulent genes found in other pathogens, such as *E. coli* or *Streptococcus pyogenes* (responsible for causing strep throat). By simply changing the temperatures and times used for the qPCR process, as well as the primers, students can easily set up methods for other qPCR experiments. The possibilities are endless! MSSU has wonderful facilities for performing scientific research.

During medical or graduate school, I would be interested in exploring other virulent genes used by *S. mutans* to contribute to dental caries. For instance, which genes does *S. mutans* use to attack other bacterial species in the oral cavity? I want to measure their concentrations in a larger population of patients. In addition, there is still so much to learn about the compositions of biofilms created by *S. mutans* to survive in the oral cavity. These biofilms are made up of numerous chemicals and have intricate structures. It would be particularly interesting to study genes used to form these biofilms. Overall, I greatly enjoyed working on my Senior Thesis. It taught me numerous valuable skills that will benefit me in my undergraduate and medical studies, as well as in my medical career.

Acknowledgements

This research was well supported by Dr. Gerald Schlink, Ms. Stacie Scrivner, Dr. Tia Strait, Ms. Katherine Corum, Dr. Lezlie Cantrell, and the Missouri Southern State University Honors Program. Thank you to the dental hygienists at the Missouri Southern State University Dental Hygiene Clinic who provided the patients' samples.

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