Original Article

The Effect of Bacteriophage on Gene's expression Responsible for Biofilm Formation in *Streptococcus mutans*

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Abstract

Background: The formation of biofilms has the potential to make bacteria resistant to antibiotics and to make immune systems reject organisms that live in them. The acids produced by bacteria that form biofilms eat away at tooth enamel, leading to cavities and other dental problems. It is new to control and prevent bacterial biofilms using bacteriophages, which are viruses that infect and kill bacteria. **Objectives:** Finding *Streptococcus mutans* bacteriophages in dental clinic wastewater and studying their effects on biofilm-related gene expression was the primary goal of this study. **Materials and Methods:** After collecting samples of dental plaque, *S. mutans* was isolated and identified using bacterial culture medium and molecular testing. To top it all off, we learned if the isolated *S. mutans* could biofilm. We were able to study the influence of lytic bacteriophages on biofilm-related gene expression using real-time polymerase chain reaction after isolating them from sewage water samples taken from Al-Najaf dental clinics. **Results:** In all, twenty samples (or 20%) tested positive for the presence of *S. mutans*. The capacity of the isolated *S. mutans* bacteria to produce biofilms varied. We tested 150 sewage water samples and found just one phage that targets *S. mutans*. **Conclusions:** By reducing harmful microorganisms and facilitating the development of effective antibacterial medications, bacteriophages have the potential to lessen the colonization of tooth surfaces by *S. mutans* and the creation of biofilms.

Keywords: Bacteriophage, biofilm, *gtfB* gene, *gtfD* gene, *Streptococcus mutans*

Introduction

Streptococcus mutans is the most important bacterium in the development of dental caries, and it is a key player in the early colonization process that leads to the development of the dental plaque biofilm (it creates adhesins that bind the microbe to the tooth's acquired pellicle).[1] *Streptococcus mutans* is known to cling to both the enamel's salivary pellicle and other plaque bacteria. It is a major player in cariogenic biofilm and a key player in the pathogenesis of tooth decay because of its acid-genic and acidic properties.[2] The presence of cavity-causing bacteria, such as mutans streptococci and lactobacilli, in acidic environments increases the incidence of cavities. It usually takes 6–24 months for caries to develop once *S. mutans* colonizes tooth decay.[3]

There are two functional domains in *S. mutans'* 1500 long-amino-acid GTFs. The absolute amino fraction releases the catalyst from the sucrose and carboxyl periphery, which promotes the binding of dextran. The ability of bacteria to attach to smooth surfaces or form biofilm in vitro, as well as their cariogenicity, were both identified to decrease when GTF mutant strains of *S. mutans* were used.[4] Plaque formation by *S. mutans* is facilitated by the conversion of sucrose to a sticky extracellular polysaccharide (ECP) by means of three particular glucosyltransferases (GTFs).[5] Proteins like GbpB and C are among the several glucan-binding proteins found in *S. mutans*.[6] At about 153 kDa, the GTF-C protein included 1375 amino acid residues. There were three small hydrophobic domains but overall it was hydrophilic. There was a gene called *gtfC* that was 4218

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base pairs distant from the *gtfB* gene. A third enzyme, GTF-D, was found to have the same kinetic properties as GTF-S and a molecular mass of 155 kDa. These GTFs are very similar to glucansucrase (GS) from Leuconostoc mesenteroides.[7] The quorum sensing system monitors and regulates the growth of biofilms. Two transmission mechanisms that are reliant on density are also present in *S. mutans*. According to,[8] the luxS gene encodes the QS and interspecific transmission systems. An essential component of biofilm formation is the quorum sensing system, which allows for communication between cells. for example, adherent cells have the potential to differentiate into sessile cells, which may then start the process of biofilm development. Quorum sensing in *S. mutans* is comprised of competence stimulating peptide (CSP) and the ComDE two-component signal transduction system. Triggers for *S. mutans'* CSP-ComDE system activation include acidic pH, amino acid deficiency, and oxidative stress.[9] Over the last 50 years, researchers have failed to find a way to cure or prevent dental caries. Modern medications are ineffective because they do not discriminate between different species; they kill both harmful and beneficial organisms. Therefore, novel treatment approaches to prevent and cure dental caries in clinical practice must be developed and put into place to specifically target cariogenic bacterial biofilms.[10] Using particular bacteriophages as the invading and destroying viruses is a novel way to combat and regulate the creation of biofilms by bacteria.[11]

Another therapeutic option that shows a lot of promise is bacteriophage (phage) therapy. When bacteria begin their lytic cycle, bacterial phages infect them, disrupt their metabolism, and eventually kill them. The increasing prevalence of antibiotic resistance has sparked a surge in Western interest in phage treatment.[12] The following are some of the benefits of phages: their ability to destroy biofilms, their low impact on the commensal microbiome, their ability to multiply at the infection site and disappear at the same time as the target pathogen, their low toxicity, their co-evolution with their bacterial host, and their ability to kill multi-drug-resistant (MDR) bacteria.[13]

Aim of study

We set out to find out how *S. mutans* bacteriophages may affect gene expression related to biofilm formation by isolating these pathogens from wastewater.

Materials and Methods

Deep, soft cavities were collected from 100 patients, whose ages ranged from 25 to 60. Who were visited to medical educational clinics of the College of Dentistry, University of Al-Kufa from November 2022 to March 2023. Used *S. mutans*, which was isolated. They were incubated at 37°C for 18–24 h after being placed in various mediums for sample growth. To verify *S. mutans* isolates, the Vitek 2 automated system ran 64 biochemical tests on GP-ID cards.

Ethical approval

All subjects involved in this study gave their informed consent prior to sample collection. A regional ethics panel looked into and permission form at College of Medicine, University of Babylon, Iraq, under the reference No. 3-6 on July 07, 2022.

Biofilm production

As per the protocol laid forth by Ellepola *et al.*,[14] all *S. mutans* isolates were phenotypically tested for their ability to form biofilms on Congo Red Agar.

Phage unique to the species *St. mutans*

From November 2022 to March 2023, the *Streptococcus mutans* phage was detected in 150 samples of sewage water from the Al-Najaf dental facility. The ice crystal structure of phage makes it unsuitable for storage at −20°C. Prior to processing, water samples were stored at 4°C for a duration of 7 days. Phage destruction time of 40 days is surpassed by this. Sewage bacteriophages may potentially reproduce in the cells of host bacteria by spinning the black water at 8000 rpm for 10 min to extract debris. Filter the cleansed supernatant through a 0.45- or 0.22-m membrane to eliminate any bacteria or viruses. At 4°C, transfer the liquid to a container that can withstand solvents, such as glass or plastic, and add a few milliliters of chloroform. Combine 20 mL of sterile bacteriophages broth medium and 20 mL of nutritional broth containing MgSO4 in a 250-mL culture flask with 100 mL of filtered material. Introduce 20 mL of each *S. mutans* strain into the flask using a fresh overnight host culture in broth culture at an OD600. For 1 day, place this enrichment culture in an incubator that mimics a host's conditions. Before adding 1 M NaCl to a 15 mL sterile Falcon tube, transfer 10 mL of enrichment cultures. To the cleaned crude lysate, add 0.5 mL of chloroform after incubating it at 2000 rpm for at least 24 h. Spin the culture in a centrifuge at 4000 rpm for 10 min. To destroy microorganisms, heat the filtered supernatant. The process of isolating bacteriophages and storing them at 4°C is described in several studies worldwide.[15-17]

Inhibitory effects of isolated lytic phages on *S. mutans* biofilm formation

To begin, *S. mutans* isolates were tested for their biofilm-forming ability at 550 nm using the microtiter plate method. The next thing we did was check whether *S. mutans* biofilm growth might be prevented by isolated lytic phages. To do this, 50 µL of phage and 100 µL of a 0.5 McFarland standard *S. mutans* solution in TSB with 2% sucrose were added to the wells of a microtiter plate. The next step was to incubate the plate with CO2 at 37°C for a full

day. After three washes, the plates were incubated at 37°C with 120 rpm shaking for 15 min. Apply 1% crystal violet (Merck, Germany) dye once they have dried. At 550 nm, the optical density (OD) of each well was measured using an ELISA reader. The tests were repeated three times each. The percentage of biofilm development was calculated using this formula:

$$\text{Equation} = \left[(C-B)-(T-B) / \ (C-B) \right] \times 100$$

This equation indicates the mean optical density (OD) of the test wells, where C is the control wells' mean OD, B is the negative controls' mean OD, and T is the test wells' mean OD.[17]

RNA extraction

We used a TRIZOL kit according to the manufacturer's instructions to extract RNA from bacterial cells: One milliliter of *S. mutans* bacterial culture broth was centrifuged at 12,000 rpm and 4°C for 1 min in an Eppendorf tube to produce the bacterial cell pellets. After adding 1 mL of TRIzol reagent, the bacterial cell pellets were shaken for 1 min. Following that, 200 µL of chloroform was added to each tube and shaken violently for 15 s. For 5 min, the ingredients were chilled. Centrifugation was also performed on it for 15 min at 4°C at 12,000 rpm. In a fresh Eppendorf tube, combine 500 µL of isopropanol with the supernatant. Flip the tube over four or five times to mix the contents. Let sit at 4°C for 10 min. In a centrifuge set at 12,000 rpm and 4°C for 10 min. Once the liquid was extracted, 1 mL of 80% ethanol was added and the mixture was vortexed once more. After that, it went through a 5-min centrifugation run at 12,000 rpm and 4°C. The RNA pellet was air-dried after the supernatant was removed. The RNA pellet was dissolved in 50 µL of DEPC water, which was added to every sample. We stored the isolated RNA at −20°C until we needed it. A Nanodrop spectrophotometer was used to quantify the total RNA extracted from three strains of *S. mutans*. A Nanodrop spectrophotometer can measure absorbance at 260 and 280 nm to assess RNA purity, since RNA absorbs the most light at that wavelength. You can tell whether DNA or RNA is pure by comparing the ratio of these two wavelengths. An RNA ratio of 2.0 and a DNA ratio of 1.8 are often considered "pure" in the scientific community. Protein, phenol, or other impurities that absorb heavily at or around 280 nm might be present if either ratio is much lower. The genomic DNA was extracted from the eluted total RNA using the DNaseI enzyme. This process was based on the promega, USA protocol.

cDNA synthesis

To construct a 20µL RT-qPCR reaction solution, the following ingredients were combined during the cDNA synthesis stage with total RNA samples processed with DNase-I. The master amplification reaction was obtained from ABM in Canada.

One microliter of forward primer (F). A reverse primer volume of 1.0 L. Combine 10µL of the mixture with 1X qPCR master Mix Buffer. Replace step 10.

There are 0.4µL of RT mix Buffer (50X) and the concentration is 1X.

Water that is, devoid of 5.56 molars. The volume of 2.0µL is the isolated RNA template.

Next, the solution is heated in a real-time PCR cycler to determine the cycle Threshold (CT) value.

Detection of gene expression of some *St. mutans* virulence genes

cDNA (extract from bacterial cells) was used as a template in Real-time PCR cycler for the detection of gene expression in some of *St. mutans* virulence genes. The primers used for the amplification of a fragment gene are listed in Table 1.[18]

RT-PCR is used for quantification of the levels of gene expression. The measured CT values during thermal reaction are recorded to compute the following measurements.[19]

Data analysis of qRT-PCR

Relative quantification of gene expression levels (fold change) was used to examine the data findings of q RT-PCR for the target gene.[20] In order for the results of a q RT-PCR experiment to have any biological

Table 1: The primers, sequences, and PCR conditions for detection of some virulence gene

Gene name	Primer sequence (5′–3′)	Size (bp)	Conditions
gtfD	F TTGACGGTGTTCGTGTTGAT R AAAGCGATAGGCGCAGTTTA	219	95°C, 5 min 95°C, 45 s 50°C, 45 s 72°C, 1 min 72°C, 10 min
gtfB	F AGCAATGCAGCCAATCTACAAAT R ACGAACTTTGCCGTTATTGTCA	96	95°C, 5 min 95°C, 45 s 50°C, 45 s 72°C, 1 min 72°C, 10 min
16S rRNA (HKG)	F CCTACGGGAGGCAGCAGTAG R CAACAGAGCTTTACGATCCGAAA	101	95°C, 5 min 95°C, 45 s 50°C, 45 s 72°C, 1 min 72°C, 10 min

Table 2: CT values required for relative quantification with reference gene as the normalizer

Genes	Test	Calibrator (cal)
Target gene	CT (target, test)	CT (target, cal)
Reference gene	CT (ref, test)	CT (ref, cal)

significance, the relative quantification approach requires the amounts to be normalized. To determine relative expression levels, this technique divides each normalized target value (CT value) by the calibrator normalized target value, which is one of the experimental samples (control samples) [Table 2]. Afterwards, the following equations were utilized to apply the ΔCT method using a reference gene:

ΔCT (calibrator) = CT (target, calibrator) – CT (ref, calibrator) ratio (target/ reference) = $2^{CT\ (reference) - CT\ (target)}$

First, normalize the CT of the reference (ref) gene to that of the target gene, for calibrator sample:

Second, normalize the CT of the reference (ref) gene to that of the target gene, for the test sample:

ΔCT (test) = CT (target, test) – CT (ref, test)

ΔΔCT= ΔCT (test) – ΔCT (calibrator)

Fold change = $2^{-\Delta\Delta CT}$

Ratio (reference/target) = $2^{CT\ (reference) - CT\ (target)}$

So, the relative expression was divided by the expression value of a chosen calibrator for each expression ratio of the test sample.

Results

One hundred participants, drawn from patients of the medical educational clinic at the College of Dentistry and the University of Al-Kufa, provided samples of soft deep caries lesions between November 2022 and March 2023. Various mediums were used for the growth of the materials. The next 18 to 24 h were spent in an incubator set at 37°C. Biochemical tests, colony shape, and microscopy led to the discovery of *S. mutans*. Staphylococcus mutans accounted for only 20% of the positive clinical samples out of 100. On GP-ID cards, the Vitek 2 automated system ran 64 biochemical tests. The method successfully and quickly identified 20 bacterial isolates with a probability ranging from 94% to 99.7%. Table 3 presents outcomes.

Table 3: Isolation and identification of *Streptococcus mutans* by automated Vitek 2 system

No. of samples	Automated Vitek 2 system	
	St. mutans	Other types of bacteria
100	20 (20%)	80 (80%)

Table 4: Biofilm formation of *Streptococcus mutans* isolates

Bacterial isolates (no.)	Biofilm formation	
	Strong	Moderate
St. mutans (20)	9 (45%)	11 (55%)

Bacterial biofilms produce chronic illnesses that are hard to cure, according to previous study. So, to differentiate between Streptococcus mutans isolates that biofilmed and those that didn't, ELISA was used in this investigation. To achieve this, we used the following median values of OD at 570 nm: > 0.240, 0.120, and 0.120. The results are shown in Table 4. Nine of the twenty isolates (or 45%) were found to be strong biofilm producers, while eleven (or 55%) were found to be moderate biofilm producers.

Phage treatment is an innovative and potentially useful method for treating and managing pathogenic bacteria. The purpose of this study was to identify any potential relationship between the reduction of *S. mutans* biofilm development and the suppression of gene expression associated with biofilm creation using bacteriophages extracted from sewage water from dental clinics in Al-Najaf city. Previously reported on the extraction of *S. mutans* phages, accomplished the same for *S. mutans* phages in saliva. However, sewage was used in this instance to isolate phages. We tested 150 sewage water samples and found just one phage that targets *S. mutans*. When separating bacteriophages, it is essential to use the correct source. Based on our findings, sewage water from dental offices is the way to go. The inquiry uncovered lytic bacteriophages that were engineered to attack *S. mutans*. We found that recombinant bacteriophage could prevent *S. mutans*, a cariogenic bacterium, from forming biofilms. Two genes, *gtfB* and *gtfD*, are associated with biofilm formation; we assessed their expression in all strains. Compared to the pure Streptococcus strain, there was a notable decrease in the levels of *gtfB* and *gtfD* transcription. The goal of this study was to identify the mechanisms by which bacteriophages influence biofilm formation by altering bacterial gene expression. Our results show that lytic bacteriophages considerably reduced gene expression. In accordance with prior phenotypic, which were validated using the microtiter plate technique. The impact of bacteriophages on the biofilm-forming capacity of *S. mutans* was congruent with our molecular data (gene expression). Taken together, the enzymes found in phages have the potential to target both planktonic and biofilm-forming bacteria in the mouth, therefore inhibiting the creation of oral biofilms [Figures 1–4; Table 4–6].

Discussion

Plaque, bacteria, and sugar all have a role in the development of dental caries, which is characterized as a chronic illness. The disease process really begins with dental plaque, a bacterial biofilm that coats the teeth. However, hard dental tissues may show signs of carious demineralization.[21] Sugar intake, saliva makeup, teeth brushing frequency, and fluoride exposure are all potential variables. The prevalence of *St. mutans*, a kind of bacterium with distinctive characteristics and a

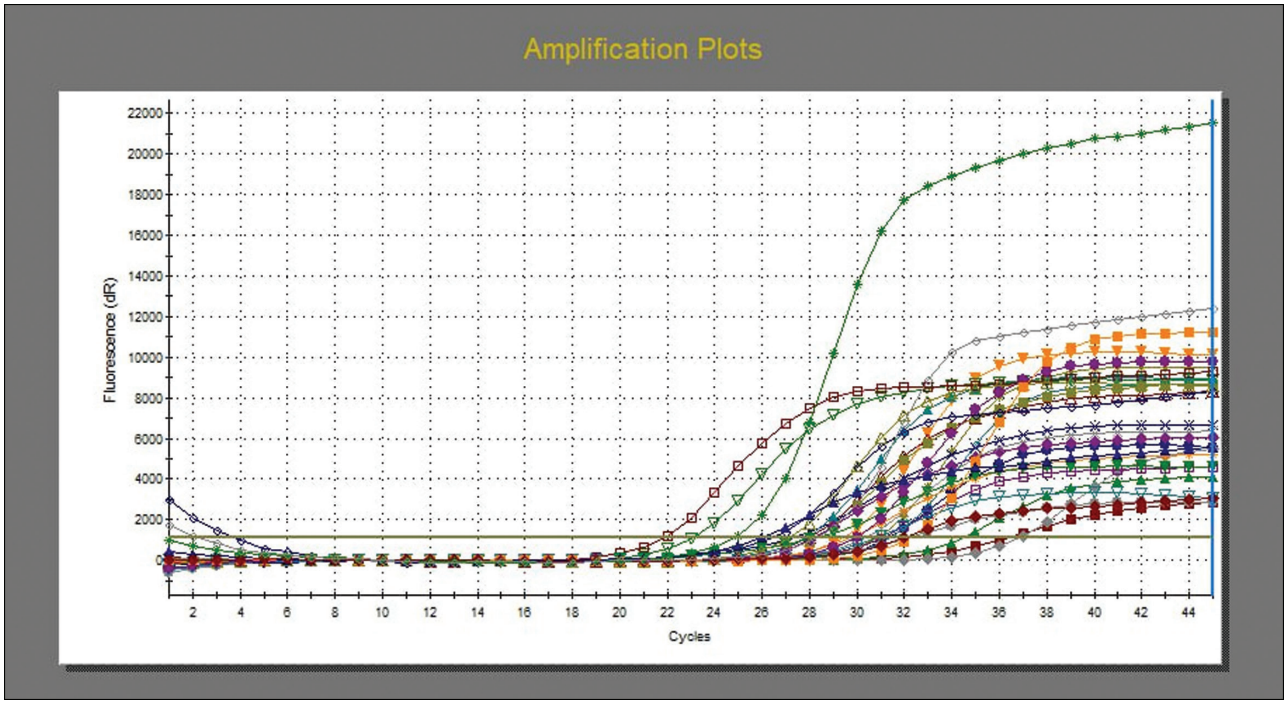

Figure 1: SYBR GREEN real-time PCR amplification chart for *gtfD* gene among 10 *Streptococcus mutans* isolates from deep dental caries lesion and 10 of the same isolates treated with bacteriophage. (Amplification plots represent the accumulation of product over the duration of real-time PCR)

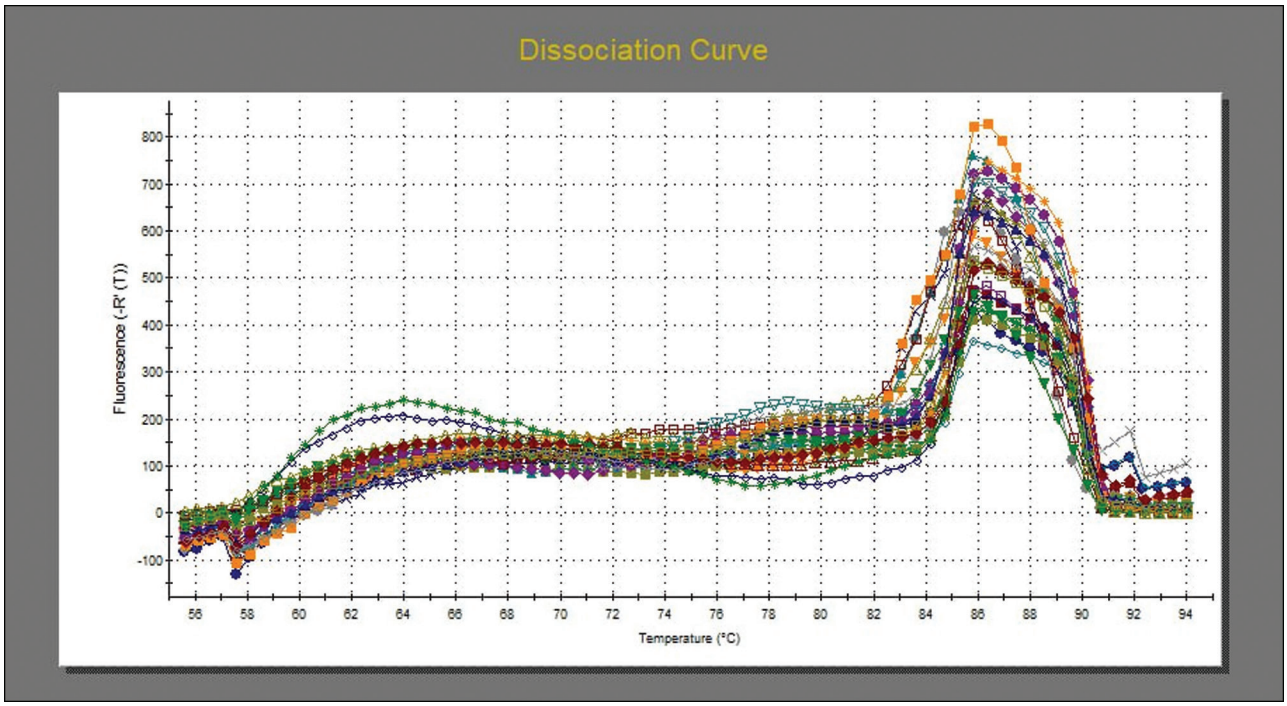

Figure 2: SYBR GREEN real-time PCR dissociation curve targeting *gtfD* gene for 10 *Streptococcus mutans* isolates from deep dental caries lesion and 10 of the same isolates treated with bacteriophage.

role in the development of dental caries (tooth decay), was found to be 25% in this study, which is in line with other findings. Mosaddad *et al.*,[22] also came to the same result. Dental caries may have several causes, including poor oral hygiene, specific microorganisms in the mouth, and dietary factors.[23] Research by Hussein *et al.*[24] has shown that Staphylococcus mutans is famous for having a metabolism that produces acid.

The purpose of this study was to identify any potential relationship between the reduction of *S. mutans* biofilm development and the suppression of gene expression associated

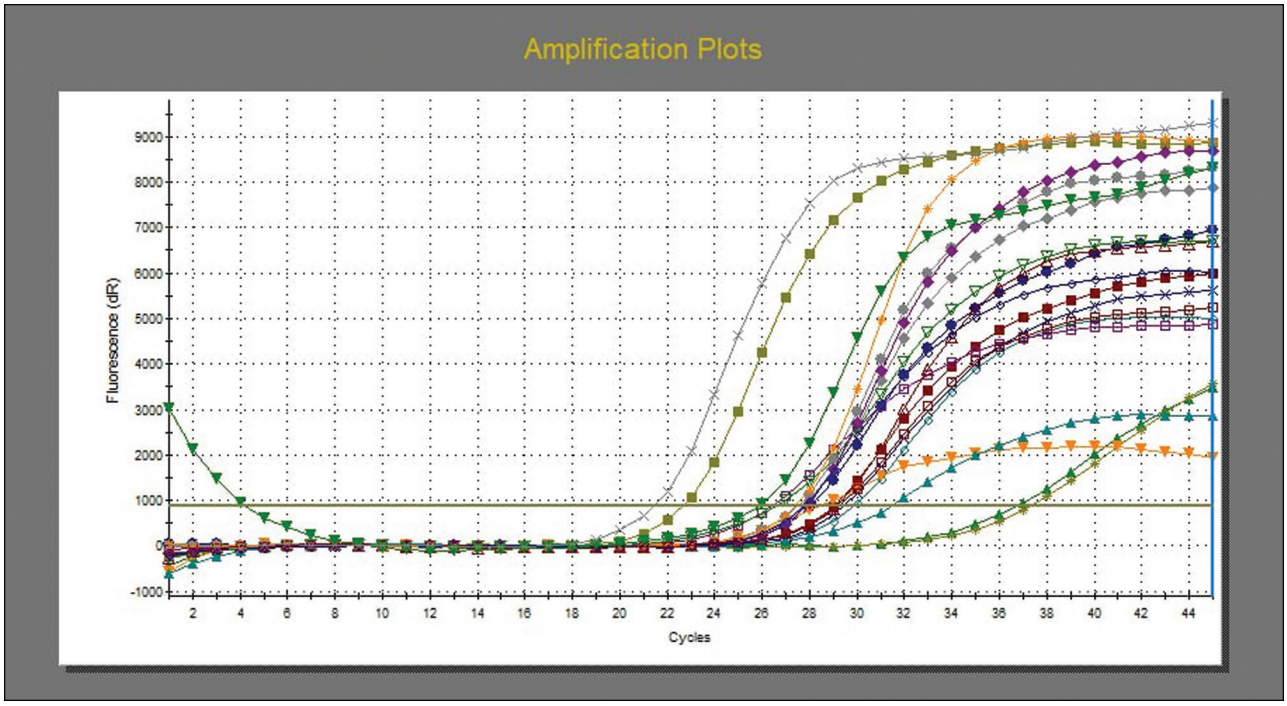

Figure 3: SYBR GREEN real-time PCR amplification chart for *gtfB* gene among 10 *Streptococcus mutans* isolates from deep dental caries lesion and 10 of the same isolates treated with bacteriophage. (Amplification plots represent the accumulation of product over the duration of real-time PCR.)

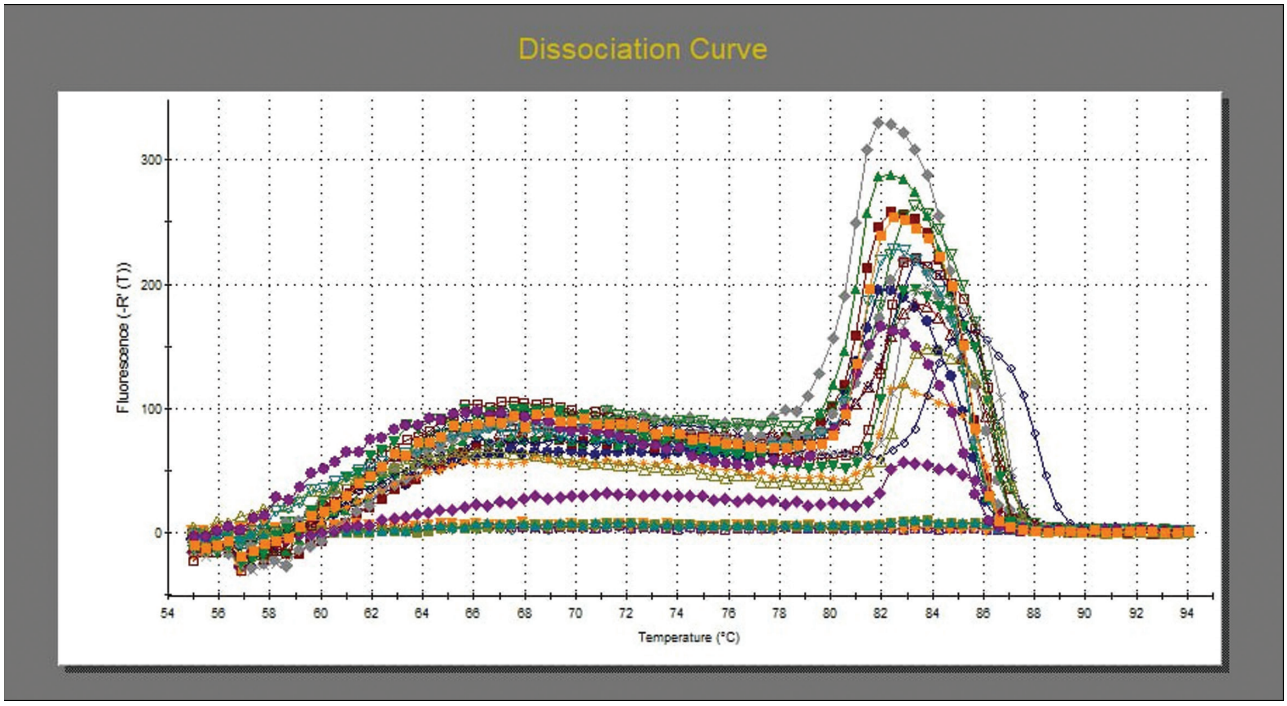

Figure 4: SYBR GREEN real-time PCR targeting *gtfB* gene for 10 *Streptococcus mutans* isolates from deep dental caries lesion and 10 of the same isolates treated with bacteriophage

with biofilm creation using bacteriophages extracted from sewage water from dental clinics in Al-Najaf city. Previously reported on the extraction of *S. mutans* phages, accomplished the same for *S. mutans* phages in saliva. However, sewage was used in this instance to isolate phages.[25] We tested 150 sewage water samples and found just one phage that targets *S. mutans*. Phage isolation from the mouth is infamously difficult, which is probably why no *S. mutans*-targeting phage was identified from saliva.[26] Our results show that lytic bacteriophages considerably reduced gene expression. In accordance with prior phenotypic results by,[27] which were validated using the microtiter plate technique. The impact of bacteriophages on

Table 5: Expresssion of *gtfD* gene before and after treated the isolates with bacteriophage

Bacterial isolates	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	CT	HKG	DCT	DDCT	2^-DDCT
S. mutans isolates	31.58	31.98	30.42	30.01	31.58	30.64	35.61	30.58	31.03	31.9	31.533	26.53	5.003	0	1
St. mutans isolates treated with bacteriophage	24.54	25.58	26.89	21.55	25.97	22.75	26.71	26.96	25.02	25.27	25.124	25.95	-0.826	-5.829	0.01759

CT = average

16S rRNA or whatever = average of housekeeping gene HKG for each *Streptococcus mutans* isolates and *Streptococcus mutans* with treated bacteriophage

DCT = CT-HKG for average of each treatment and not treated samples

DDCT or (−DCT) = DCT–DCT of control

Table 6: Expresssion of *gtfB* gene before and after treated the isolates with bacteriophage

Bacterial isolates	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	CT	HKG	DCT	DDCT	2^-DDCT
St. mutans isolates	27.17	26.6	26.44	26.82	26.91	26.61	26.93	26.58	26.93	27.69	26.868	21.23	5.638	0	1
St. mutans isolates treated with bacteriophage	26.82	25.27	23.09	21.23	25.98	24.41	23.3	21.28	21.37	22.89	23.564	20.25	3.314	-2.324	0.19971

the biofilm-forming capacity of *S. mutans* was congruent with our molecular data (gene expression). Taken together, the enzymes found in phages have the potential to target both planktonic and biofilm-forming bacteria in the mouth, therefore inhibiting the creation of oral biofilms. Numerous studies, concentrating on preschoolers, have examined the relationship between *S. mutans* and the tooth decay index, both in the United States and elsewhere. Among children less than 7 years old, Yang *et al.*[28] observed that *S. mutans* was the most prevalent oral streptococcal infection linked to dental caries. In line with other studies, our findings show that the presence of *S. mutans* in the mouth significantly increases the probability of dental plaque formation. Disparities in *S. mutans* prevalence estimates are most likely caused by variations in the genetic makeup, cultural practices, and dietary habits of people from different regions of the globe. It is believed that phage treatment is an effective method for treating and controlling pathogenic microbes.[29] In order to lessen the biofilm development by *S. mutans*, this study sought to identify if bacteriophages isolated from urban wastewater might inhibit the expression of genes involved in biofilm generation. Rajabi *et al.*[30] recovered *S. mutans* phages from Swage waters, while Bachrach *et al.*[31] isolated *S. mutans* phages from saliva. However, our work successfully isolated phages from raw sewage. We were able to identify a phage against *S. mutans* from saliva, which is surprising considering how difficult it is to separate phages from the oral cavity. Our study indicates that raw urban sewage might be the most suitable source for isolating bacteriophages. This work uncovered lytic bacteriophages that were engineered to target *S. mutans*.[32]

Conclusions

Bacteriophages may hold the key to a new approach to developing safe antibacterial drugs and preventing the spread of infectious diseases. Based on our results and previous studies, phage therapy seems to be a good way to reduce biofilm formation and decrease *S. mutans* colonization of tooth surfaces.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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