ISSN 2462-2052 | eISSN 2600-8718 *Journal of Science and Mathematics Letters*
DOI: https://doi.org/10.37134/jsml.vol12.2.5.2024 *Volume 12, Issue 2, 74-82, 2024*

Research Article

Detection of *SpaP* and *vicR* Genes in Non-biofilm Producing *Streptococcus mutans* Isolated from Dental Caries and Plaque

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Received: 18 February 2024; **Accepted:** 2 April 2024; **Published:** 15 May 2024

ABSTRACT

Evidence accumulated over many decades has clearly shown that *Streptococcus mutans* is a major agent in dental caries. *S. mutans SpaP* gene (Cell Surface Antigen) mediates the binding of these cariogenic bacteria to tooth surfaces. This study aimed to detect the presence of *SpaP* and *vicR* genes in non-biofilm-forming *S. mutans* isolated from dental plaques. In this study, 120 clinical samples were obtained from different dental clinics and hospitals in Baghdad. 26 bacterial isolates were identified as *S. mutans* by using selective media (Mitis-salivarius agar) and microscopic examination after gram staining then confirmed *S. mutans* by molecular detection using *SpaP* genes. Interestingly, all 26 isolates were non-biofilm forming after microtiter plate assay based on the crystal violet staining method, and negatively resulted in the detection of *vicR* gene which is responsible for biofilm formation. Ten antibiotics (doxycycline, ofloxacin, tetracycline, erythromycin, vancomycin, clindamycin, rifampicin, imipenem, amikacin and cefepime) were used to examine the sensitivity of *S. mutans* isolates, all isolates were multi-drugs resist (MDR) for all 10 antibiotics used. We can conclude that the biofilm-forming ability has no major role in the antibiotic resistance mechanism or virulence of *S. mutans*.

Keywords: non-biofilm producing *Streptococcus mutans*, *SpaP* gene, *vicR* gene, dental plaque, multidrugs resist

1. INTRODUCTION

One of the most prevalent health issues in the world, dental caries, is a chronic infectious illness that affects mineralized tooth tissue and places a significant financial, social, and health-related burden on society (Zou et al., 2022). *Streptococcus mutans* is a Gram-positive bacterium that is a common inhabitant of the mouth cavity in humans. It is known to be a major cause of dental caries or tooth decay, making it an important target for dental research and treatment (Iacopetta et al., 2023). *S. mutans* belongs to the genus *Streptococcus*, which is a diverse group of bacteria that are characterized by their spherical or ovoid shape and their ability to form chains. Within the genus *Streptococcus*, *S. mutans* is classified as a member of the mutans streptococci, which also includes *Streptococcus sobrinus*, *Streptococcus cricetus*, and *Streptococcus downei* (Suvarna and Mahon, 2022). *S. mutans* is a facultative anaerobe, meaning that it can survive and grow in both the presence and absence

of oxygen. It is also acidogenic and acid-tolerant, which means that it can produce and withstand acidic conditions, making it particularly well-suited to survive in the oral environment. It produces several virulence factors, such as surface proteins, extracellular polysaccharides, and sugar-degrading enzymes, that enable it on colonize and cling to teeth (Alejandra and Daniel, 2020). Cavities develop as a result of the erosion of the enamel and dentin of teeth caused by acids produced by oral bacteria. *S. mutans* is particularly well adapted to this process, as it is able to metabolize dietary sugars into lactic acid, which can then dissolve tooth enamel. It also produces extracellular polysaccharides that allow it to form biofilms, which protect it from the immune system and other antimicrobial agents (Karygianni et al., 2020). *S. mutans* is a vital component of the oral microbiota and is crucial for the emergence of dental caries. Its ability to colonize and survive in the oral environment, along with its acidogenic and acid-tolerant properties, makes it a key target for research into the prevention and treatment of dental caries (Bedoya-Correa et al., 2019; Aljubouri and Alobaidi, 2023). A conserved sucrose-independent adhesin called *SpaP* is one of *S. mutans*' essential adhesion and colonization factors. P1, Antigen I/II, and Pac 3 are other names for *SpaP* (Yang et al., 2019a). By specifically interacting with salivary agglutinin (SAG), a salivary component, it facilitates the adhesion of *S. mutans* to the saliva-coated tooth surface (Manzer et al., 2020). Therefore it's important to isolate the *S. mutans* from different dental caries and plaques from clinical samples in Iraq, and detect the presence of *SpaP* and *vicR* genes in non-biofilm forming *S. mutans* isolated from dental caries and plaques.

2. MATERIALS AND METHODS

2.1. Samples Selection and Collection

Samples of dental plaques and tooth caries were taken from 120 people (ranging from 4 to 67 years old) from Dental Specialist Center Sheikh Omar and Mahmoudia Specialized Dental Center in Baghdad, Iraq. These samples were directly put in a tube, which contained normal saline transferred to a laboratory within a maximum period of 1-5 h, and then it is culture in brain heart infusion broth for 24 h. After the incubation period, a loopful culture was streaked on Mitis Salivarus agar media and then incubated at 37°C overnight. A single colony was picked up with a sterile loop, transferred to a brain heart infusion medium, and cultured at the same temperature. This subculturing process was repeated repeatedly until a single pure colony was transferred into brain heart infusion tubes. This bacterial growth was used for molecular PCR identification. After molecular PCR identification and antibiotic sensitivity test (AST) was made, only three microorganisms were chosen to test the plant product on it.

2.2. Short Term Preservation

After the insurance of its diagnosis, bacterial isolates were kept by transferring a single pure isolated colony to BHI agar slant , incubated overnight at 37ºC, then stored in the refrigerator at 4°C for a short period of time (Maniatis, 1982).

2.3. Morphological Characterization

2.3.1. Gram Stain Test

According to the manufacturing firm Syrbio/Switzerland, this operation was carried out. A drop of sterile water was applied to the slide using a sterile cooling loop. A loopful of a bacterial colony was carefully combined with a drop of water using a cleaned and cooled loop

to create an emulsion. The slide was held at one end and ran over the Bunsen burner flame twice to three times with the smear side up to fix bacterial cells. A staining tray was used to slowly saturate the crystal violet on the slide for one minute. It was mildly and gently cleansed with tap water and a wash bottle. After that, it was immersed in iodine for a minute. It was then given a gentle wash with tap water. Drop by drop, a decolorizing chemical was applied to the slide for 5 to 10 sec, and then the slide was promptly cleaned with water. Safranin was then gradually poured into the mixture for 45 sec. The slide was carefully cleaned with tap water. The slide was dried using the filter paper before being inspected under a 40× light microscope.

2.3.2. Biofilm and Growth Assays Preparation

We investigated the ability of *S. mutans* to form biofilms using the component crystal violet staining method described by (Ahn et al., 2008). O'Toole and Kolter (198) was the first used this technique to find mutants lacking in biofilm, as the most often used quantitation method in microplate assays, despite its drawbacks and the development of more complex approaches (Azeredo et al., 2017). Each well of the polystyrene 96-well flat-bottom microtiter plates used for the experiments included 20 mL of suspended bacteria with a 0.5-0.7 McFarland concentration ($1.5*10^8$ cfu/ml) as the inoculum, along with 180 mL of Mueller-Hinton broth that was supplemented with 1% glucose. At 37°C, the microplates were incubated for 24 h. After twice rinsing the adhering cells in phosphate buffered saline (PBS), the wells were dried at 60°C for no more than an hour. The liquid media was discarded, 150 μL of 2% crystal violet was then used to stain it for 15 min. The microplate wells treated with crystal violet were then rinsed twice with PBS to remove the stain.

Following the air drying of the microplate's wells, 150 μL of 95% ethanol are used to re-solubilize the dye from the biofilms that lined the plate's walls. A microplate reader measures the microplate spectrophotometrically at 570 nm. At least three fresh samples were used in the experiment each time (Eydou et al., 2020). The optical density cut-off value (ODc) was determined as three SD above the average of the negative control's optical density (OD), as calculated using the formula; ODc = average OD of the negative control + (3×SD of negative control). The studies are repeated in triplicate. Uninoculated wells containing sterile MHB supplemented with 1% glucose that are considered negative controls are used as blanks. The results were separated into four groups based on their optical densities: strong biofilm producer ($4\times ODc<OD$), medium biofilm producer ($2\times ODc<OD\leq 4\times ODc$), weak biofilm producer ($ODc<OD\leq 2\times ODc$), and non-biofilm producer ($OD\leq ODc$) (Stepanovic et al., 2007).

2.4. Antibiotic Sensitivity Test

Synthetic antibiotics were tested against pathogenic microorganisms, namely, *S. mutans* using the Mueller Hinton Agar. The disk diffusion methods were used as well as the antibiotics (doxycycline, tetracycline, vancomycine, amikacine - 30 mcg, ofloxacine, rifampicine - 5 mcg, erythromycine - 15 mcg, clindamycine - 2 mcg, imipeneme, cefepime - 10 mcg). On the petri dish, the pathogenic bacteria were streaked. At 37°C, the petri dishes were incubated. After 24 h of incubation, for each microbial culture, the inhibition zones were measured in mm (Gentile et al., 2014).

2.5. Molecular Experimental Studies

2.5.1. DNA Extraction

The following procedures were used to extract genomic DNA from bacterial growth using the ABIOpure Extraction protocol: The cultures spin in 1 mL for 2 min at 13,000 rpm to

obtain pellet cells. The excess liquid was then discarded. Then the sample was added to a tube along with 20 µL of Proteinase K solution (20 mg/mL) and 200 µL of buffer BL. The tube was then forcefully agitated using a vortex and incubated at 56°C for 30 min. Then added to the sample, 200 µL of 100% ethanol and the sample was thoroughly mixed using a pulse vortex. After carefully transferring each mixture to a small column, it was centrifuged for one minute at 8,000 rpm, and the collecting tube was changed for a fresh one. After that, 600 µL of buffer BW were added to the small column, which was then centrifuged for one minute at 8,000 rpm with a fresh collecting tube. TW 700 µL was applied to the buffer. 8,000 rpm for 1 min while centrifuging. The mini-column was reinserted into the collection tube after the pass-through was discarded. The mini-column was centrifuged at full speed for 1 min (13,000 rpm) to remove any remaining wash buffer, and it was then put into a new 1.5 mL tube. Finally, 60 µL of buffer AE was added, let to sit at room temperature for 1 min, and then centrifuged for 5 min at 5,000 rpm.

2.5.2. Quantitation of DNA

The concentration of extracted DNA was measured using a Quantus Fluorometer to determine the sample quality for subsequent use. 200 µL of diluted Quantifluor Dye were combined with 1 µL of DNA. DNA concentration readings were found following a 5 min incubation period at room temperature. The Macrogen Company provided these primers in lyophilized form. As a stock solution, lyophilized primers were dissolved in nuclease-free water to a final concentration of 100 pmol/µL. 90 µL of nuclease-free water was combined with 10 µL of primer stock solution (kept at freezer -20°C) to create a usable primer solution containing 10 pmol/µL of these primers (Table 1).

Table 1. Primer preparation

Primer Name	Vol. of nuclease free water (µl)	Concentration (pmol/µl)
spaP-F	300	100
SpaP-R	300	100
vicR-F	300	100
vicR-R	300	100

2.6. Reaction Setup and Thermal Cycling Protocol

2.6.1. PCR Amplification

Isolated genomic DNA obtained from the bacterial isolate was used as a template for PCR. SpaP and vicR genes were amplified. The PCR reaction was performed by adding the following: 10 µL of GoTaq® G2 Master Mix, 4 µL of template DNA (genomic DNA), 1 µL of each of the upstream and downstream primers and 4 µL nuclease-free water to complete the volume to 20 µL. The reaction program consisted of the following steps: a first phase of 5 min of denaturation at 95°C, then 30 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. There was also a final extension phase lasting 7 min at 72°C (Kim et al., 2015).

2.6.2. Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to verify the existence of amplification following PCR amplification. The criteria based on the isolated DNA were totally reliable for PCR. Solutions - DNA ladder marker, 1×TAE buffer, and Ethidium bromide (10 mg/mL). Preparations of agarose - 100 mL of 1X TAE in a flask were taken. The buffer received 1.5 gm (for 1.5% agarose concentration). The solution was microwaved to boiling, when all gel particles had been dissolved. To the agarose, 1 µL of ethidium bromide (10 mg/mL) was

added. To combine the agarose and prevent bubbles, it was stirred. At 50-60°C, the solution was allowed to cool. Casting of the horizontal agarose gel - After sealing the edges with cellophane tape on both sides, the agarose solution was poured into the gel tray, where it was allowed to set for 30 min at room temperature. The gel was carefully placed in the gel tray after the comb had been removed. 1X TAE-electrophoresis buffer was poured into the tray until it was 3-5 mm above the gel's surface. DNA loading - The PCR products were directly loaded. 5 µL of the PCR product were added straight to the well. 100 v/mAmp of electricity was turned on for 60 min. From the cathode to the positive anode poles, DNA travels. Using the gel imaging equipment, the ethidium bromide-stained bands in the gel were seen.

3. RESULTS AND DISCUSSION

In this study, 120 clinical samples were obtained from different dental clinics and hospitals in Baghdad. 26 Bacterial isolates were identified as *Streptococcus mutans* by using selective media (Mitis-salivarius agar) and microscopic examination after gram staining. *S. mutans* are gram +ve (Figure 1). The colony morphology from direct visualization and light microscopic observation are having colonies that were round or spherical in shape and about 2-3 mm in size, appeared pale blue, and were raised or convex and tightly adhered to the surface of the selective media agar plates (Figure 2).

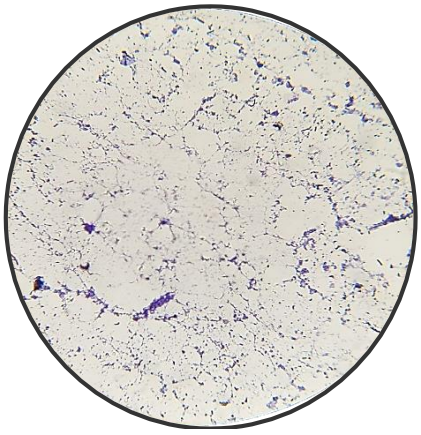

Figure 1. *S. mutans* under light microscope after gram stained

Molecular identification of 26 bacterial isolates (Figure 3) which were selected on selective media (Mitis-salivarius agar) and microscopic examination after gram staining, confirmed *S. mutans* by molecular detection of *SpaP* genes using PCR and specific primer recognized specific sequences within *SpaP* genes associated with *S. mutans* (Yang et al. 2019b).

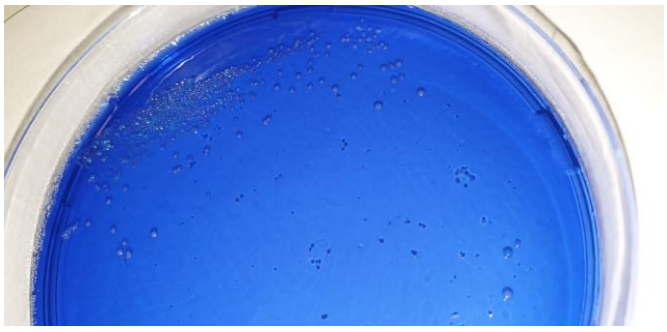

Figure 2. *S. mutans* colonies on Mitis-salivarius agar plate

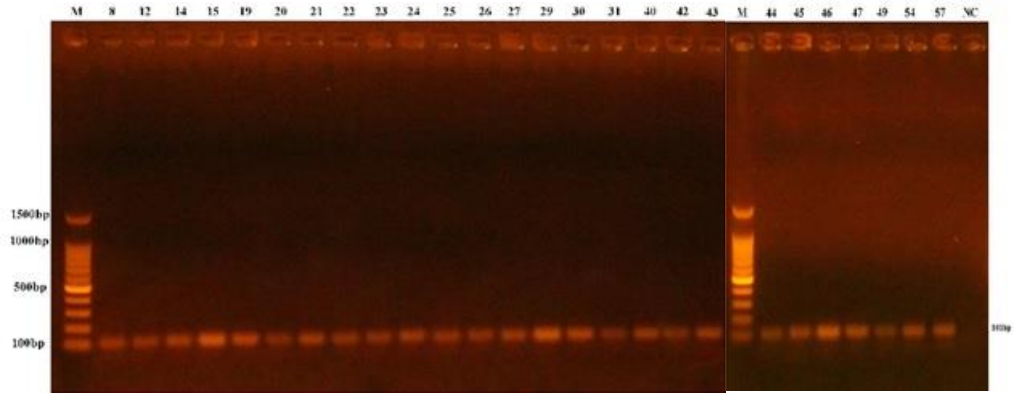

Figure 3. Results of the amplification of *spaP gene* of *Streptococcus mutans* samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 8-57 resemble 101bp PCR products

Interestingly, all 26 isolates were non-biofilm forming after microtiter plate assay based on the crystal violet staining method, and negatively resulted in the detection of *vicR* gene which responsible for biofilm formation (Figure 4).

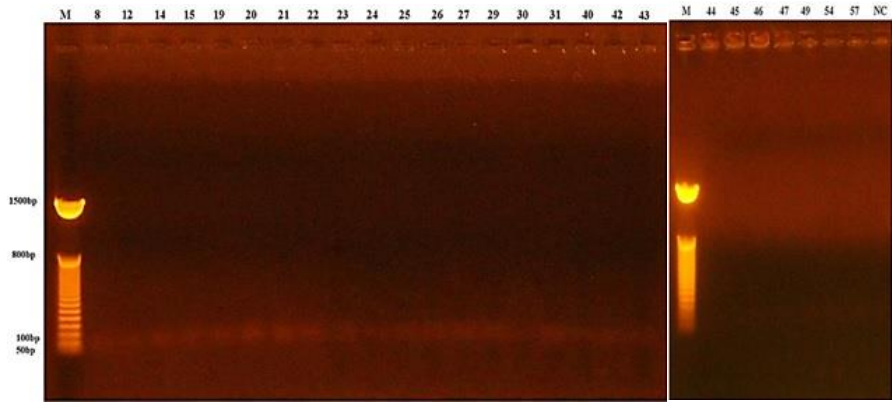

Figure 4. Results of the amplification of *vicR gene* of *Streptococcus mutans* samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 8-57 resemble 101bp PCR products

Culture-dependent methods that use MS media to isolate, cultivate, identify, and quantify *S. mutans* are commonly used in cardiology, clinical, and epidemiology studies. Moreover, limited studies have characterized and identified non-*S. mutans* microorganisms isolated from MS (Zeng et al., 2020). The mutans streptococci constitute a group of oral cariogenic species, with *S. mutans* being the most frequently detected strain in the oral cavity of individuals at high risk for dental caries (Nelun Barfod et al., 2011).

Results of the biofilm formation for all 26 Bacterial isolates identified as *Streptococcus mutans* by using selective media (Mitis-salivarius agar) were negative (Figure 5). Despite the fact that the bacterial isolates were obtained from patients with dental caries and dental plaques but these isolates lack the ability to form biofilm which is reported in many literatures (Mattos-Graner et al., 2004; Bottner et al., 2020). Dental caries and dental plaques contain more microorganisms which have the ability to form biofilm other than *S. mutans* (Valm, 2019), this could improve that the ability of *S. mutans* to form biofilm is not essential to develop the virulence activity.

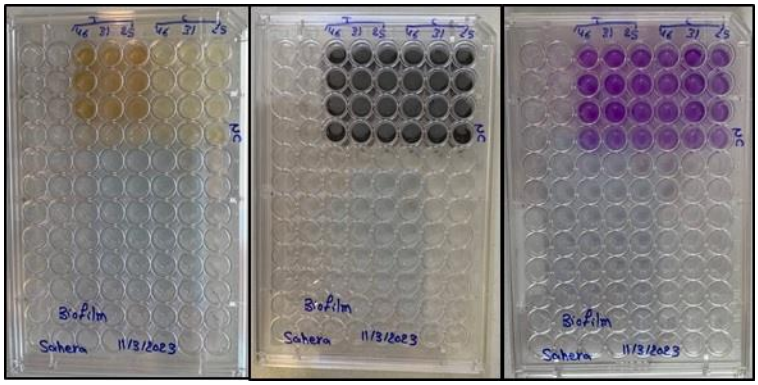

Figure 5. The ability of *S. mutans* to form biofilms using crystal violet staining method. The OD readings of the samples were (0.10 – 0.15)

Synthetic antibiotics were tested against *S. mutans* using the Mueller Hinton Agar. The disk diffusion methods were used with ten antibiotics (doxycycline 30 µg, Ofloxacine 5 µg, tetracycline 30 µg, erythromycine 15 µg, vancomycine 30 µg, clindamycine 2 µg, rifampicine 5 µg, imipeneme 10 µg, amikacine 30 µg, cefepime 10 µg) all 26 isolates were multi-drugs resist (MDR) for all 10 antibiotics used (Figure 6).

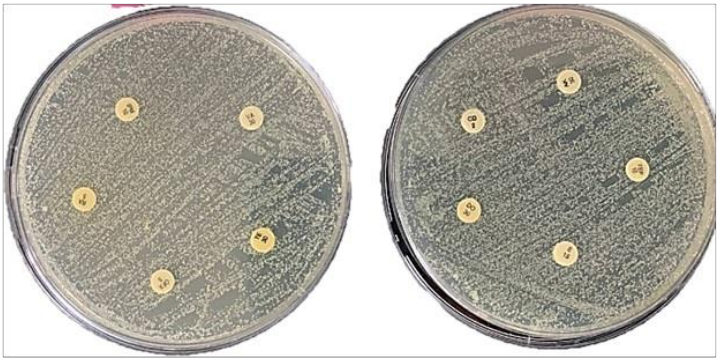

Figure 6. Antibiotic sensitivity test using the Mueller Hinton Agar. The disk diffusion methods were used, then antibiotics (doxycycline 30 µg, ofloxacin 5 µg, tetracycline 30 µg, erythromycin 15 µg, vancomycin 30 µg, clindamycin 2 µg, rifampicin 5 µg, imipenem 10 µg, amikacin 30 µg, cefepim 10 µg)

Most of the antibiotics employed in this study are commonly prescribed by dentists. The number use use of resistant of oral *S. mutans* is greater in people frequently exposed to antibiotics, although resistant bacteria may also be found in healthy subjects who have not been recently treated with antibiotics (Salman et al., 2017). β-Lactam antibiotics are the most commonly prescribed chemo prophylactic agent's in general dental practice. However, resistance to penicillin among oral streptococci is increasing (Pasquantonio et al, 2012). The number of resistant oral streptococci is greater in people frequently exposed to antibiotics, although these bacteria may also be found in healthy subjects who have not been recently treated with an antimicrobial (Sweeney et al., 2004).

4. CONCLUSION

This study highlights the presence of *S. mutans* in dental plaques and its association with dental caries. Despite the absence of biofilm formation in the isolated strains, the detection of the *SpaP* gene reaffirms their identity as *S. mutans*. Furthermore, the resistance of

these isolates to multiple antibiotics underscores concerning the trend of multidrug resistance in this pathogen. Importantly, the findings suggest that the lack of biofilm formation does not diminish the antibiotic resistance or virulence potential of *S. mutans*, emphasizing the need for alternative strategies in combating dental caries and associated infections. Further research into understanding the mechanisms underlying antibiotic resistance and virulence in non-biofilm forming *S. mutans* strains is warranted to inform more effective therapeutic interventions.

Declaration of Interest

The authors declare that there is no conflict of interest.

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