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Biofilm Production Activity and Antibiotics Susceptibility of *Streptococcus mutans* Isolates from Patients with Dental Caries

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ABSTRACT: Dental caries, a prevalent chronic infectious ailment, commences with the adherence of cariogenic bacteria to the teeth, particularly *Streptococcus mutans*. These microorganisms utilize sugars to produce acid, resulting in the gradual demineralization of the dental structure. *Streptococcus mutans* a gram-positive bacterium is found to play the most significant role in the dental caries in terms of being the earliest colonizer in the formation of caries. The current research focuses on the isolation and identification of the *Streptococcus mutans* pathogen in individuals affected by dental caries. The isolated strains were cultured on specific media, with one hundred samples of dental caries obtained from human teeth. Among these samples, fifty were identified as positive bacterial isolates utilizing Mitist Salivares agar, with ten isolates categorized under the group Streptococci and twenty isolates identified as *S. mutans*. Identification of bacterial isolates was carried out based on morphological and cultural characteristics, biochemical tests, and VITEK-II. Additionally, an antibiogram assay was conducted to assess the susceptibility and resistance of the pathogen to various drugs. The findings revealed that all locally isolated *S. mutans* strains displayed 100% resistance to Cefepime, while showing different levels of resistance and susceptibility to other antibiotics such as Doxycycline, Amoxicillin-clavulanic acid, Trimethoprim-sulfamethoxazole, Vancomycin, Tetracycline, Ampicillin, and Meropenem. In this study, ELISA was employed to differentiate between *Streptococcus. mutans* isolates that produced biofilms and those that did not by utilizing the optical density at 630 nm. Bacterial biofilms contribute to chronic diseases that pose challenges in treatment. Twenty distinct isolates were evaluated for their biofilm-forming capabilities, with 9 (45%) identified as strong biofilm producers, 7 (35%) classified as moderate biofilm producers, and 4 (20%) categorized as weak biofilm producers

Keywords: Dental caries, *Streptococcus mutans*, Biofilm prodection, antibiogram assay

1. INTRODUCTION

Dental caries stands out as a significant healthcare issue due to its global prevalence. The disease affects nearly all adults (1). It is characterized as a chronic, transmissible, and infectious microbial disease, where micro-organisms metabolize sugars from the diet to produce acid. The process of caries involves an interaction between the biofilm (known as dental plaque, consisting of resident bacteria from saliva) and the tooth surfaces. This process typically advances slowly in most individuals. The disease is infectious, commonly transmitted from mother to infant or between family members through activities involving saliva sharing (2). Dental caries lesions can emerge at any tooth site. In 2015, the global economic impact of dental diseases amounted to $544.41 billion, with seventy-nine percent attributed to severe tooth loss and untreated dental caries (3). The development of dental caries is linked to a shift in the biofilm microbiota residing in the oral cavity towards an acidogenic, aciduric, and cariogenic population, primarily due to the frequent intake of sugars (4). Consequently, dental caries is recognized as a dietary-microbial disease necessitating a cariogenic biofilm and consistent exposure to fermentable carbohydrates such as glucose, fructose, maltose, and sucrose from the diet (5). *Streptococcus mutans*, as described by Clarke in 1924, is a Gram-positive microorganism that fulfills a crucial function

in the development of dental synthesizes adhesins to facilitate its attachment to the acquired pellicle on tooth surfaces and stands out as the principal contributor to the onset of dental cavities. Unlike bacteria possessing flagella, *S. mutans* lack these structures but exhibit pili. When cultivated on agar, *Streptococcus. mutans* are visualized as gram positive ovoid cocci usually in pairs or in chains. They are quiet, acid in fact demanding acidic growth conditions as they exhibit aciduric characteristics and are capable of producing acid, that is, they are acidogenic. actively generating acid. Additionally, *S. mutans* are non-motile facultative anaerobes that exhibit optimal growth at a temperature of 37 °C. (6). The progression of dental caries is linked to the establishment of biofilms that impact a considerable portion of the global population. It is widely accepted that the bacterium *Streptococcus mutans* is the principal etiological factor contributing to this significant pathology. *S. mutans* is crucial in the creation of intricate and multi-dimensional structures on both the oral mucosa and tooth enamel (7). Said bacterium possesses certain cariogenic characteristics, including its capacity for surface adhesion, colonization of the oral cavity, and resilience to the acidic environment of the mouth (8). Moreover, *Streptococcus mutans* utilizes acidic metabolites generated from carbohydrates, resulting in acid-induced deterioration and demineralization of tooth enamel through the removal of mineral components, consequently initiating the development of dental caries. (9)

the present work was done to isolate and identify *Streptococcus mutans* from dental caries, study antibiotics susceptibility of *S. mutans* isolates and study biofilm formation by S.*mutans* isolates .

2. MATERIALS AND METHODS

2.1 Sample collection

Samples for analysis were collected by the means of swabs taken from the oral cavity of patients with certain forms of carries, including pit, fissure, and dental roots. Subsequent to collection, these samples were placed in peptone water to enriched them then streaked on to mitis salivaris agar (MSA). The individuals from whom the samples were taken were residents of Al-Kut city and had sought treatment at the Dental specialist center. Information such as name, age, diabetic status, and clinical symptoms was recorded by the attending physician during data collection.

2.2 Bacterial Isolation and Identification

2.2.1 *Streptococcus mutans* Isolation

Bacterial strains derived from samples of dental caries were streaked onto a specialized growth medium known as MSA to facilitate the isolation of *Streptococcus mutans*. Following this, the Petri dishes were placed in a controlled environment for a duration of 48 h at a temperature of 37°C, all while being subjected to anaerobic conditions within a candle jar. This process was iterated multiple times until a culture that was entirely pure was successfully achieved. (10)

2.2.2 *Streptococcus mutans* Identification

Bacterial isolates were classified based on their cultural traits and morphological (properties), as well as through biochemical assays and the VITEK-II system.

•morphological and cultural characteristics

Features related to morphology and culture Following anaerobic 48 hours at 37°C in a candle jar, the bacterial isolates' colony size, shape, and color were examined on Mitis Salvaris Bacitracin agar. Under a light microscope, the form, aggregate, and arrangement of the cells were examined.

• Biochemical tests

1- Catalase test .

With this assay, the potential of bacterial isolates in the production of the catalase enzyme involves the following steps. enzyme which transforms hydrogen peroxide into the generation of lesser amounts of oxygen and water. was determined. A growth is taken from a by means of a wooden stick applicator, a clump of growth. a loop of pure culture of each bacterial isolate was then streaked on a microscopical slide. Three milliliters of 3% hydrogen peroxide solution followed by two drops of the same was then administered. bacterial cells make gaseous bubbles as a sign of success (11) .

2- Blood hemolysis test

The bacterial isolate from each sample was grown separately streaked on Blood Agar plate. grown for 48hrs at 37°C under anaerobic conditions to identify the kind of hemolysis (12).

•Identification by using VITEK-2

identification scheme A larger identification database, the most automated platform currently in use, quick results, increased confidence and little training time are all features of the cutting-edge VITEK-2 microbial identification system. The VITEK-2 identification technology was used to fully identify bacterial isolates that were thought to be *S. mutans.*

2.3 Antibiotic susceptibility testing

The examination is conducted through the Kirby-Bauer method on Muller Hinton agar . Inoculum isolates were generated by dispersing colonies from an overnight culture in sterile normal saline until reaching a turbidity comparable to the 0.5 McFarland solution standards. The bacterial suspension was evenly distributed on Muller Hinton agar using a sterile swab and allowed to desiccate. Subsequently, a set of sterile forceps was employed to place selected antibiotic discs Penicillin G , Ampicillin , Tetracycline , Vancomycin , Erythromycin , Cefotaxime , Ceftriaxone , Doxycycline , Cefepime , Meropenem , Levofloxacin , Amoxicillin-clavulanic acid , Trimethoprim-sulfamethoxazole , Clindamycin onto the inoculated plates, followed by an incubation period of 48 hours at 37°C in an inverted orientation. Upon completion of this incubation timeframe, the diameters of the inhibition zones were recorded and measured using a ruler in millimeters (mm), with results interpretation conducted in accordance with CLSI guidelines (13) .

2.4 Testing biofilm formation by the recovered isolates

In this study, the ability of *S. mutans* to form biofilms and biofilm formation by isolates was evaluated by the crystal plate method as described by Zhou *et al* (14) and Al Quraishi *et al.* (15). Isolates were cultured in BHIA and then placed in a candle jar for 48 h at 37°C . Bacterial colonies were streaked into a tube containing isotonic saline and approximately 0.5 McFarland turbidity standards. The saline solution was then transferred to brain heart broth, 200 µl of which, and each of the diluted solutions was placed in a 96-well plate with fresh media for each well. Plates were incubated for 48 h at 36°C, while negative control wells contained all components except bacteria. This liquid medium was then discarded and the plates were washed twice with saline before a final wash with saline. Biofilms were titrated with 200 µl of 0.1% crystal violet for 15 min. Any remaining dye was washed off with distilled water and then allowed to dry for 15 min. Plates were then solubilized with 200 µl of 96% ethanol, and the density was measured. Optical panels then. Read at 630 nm on a microplate reader by enzyme linked immunosorbent assay [ELISA]. Each clinical strain was tested in triplicate, after which the data were pooled and the standard deviation calculated. The average of optical density (OD) value recorded for the control wells (containing media without inoculation) was subtracted from the average OD values for each individual test strain. The derived results were used to classify the test strains based on their biofilm-forming abilities, as shown in the table (1). a ODc is OD of the control

Table 1. OD value of Biofilm ranking

OD value	Biofilm formation
OD ≤ 2* ODc	Weak Biofilm
2* ODc ≤ OD ≤ 4 *ODc	Moderate Biofilm
4 *ODc ≤ OD	Strong Biofilm

3. RESULTS

3.1. Collection of samples

In this study, a total of one hundred specimens were acquired through the collection of swabs from individuals afflicted with dental caries. After inoculated onto specialized Mitis Salivaris agar medium (MSA). Out of the initial 100 specimens, a mere fifty exhibited favorable outcomes.

3.2. Identification of bacterial isolates

In our study bacterial strains cultivated on a specialized medium known as MSA medium and suspected to be of the species *S. mutans* were differentiated based on their morphological features, cultural properties, biochemical assays, gram staining, and the use of VITEK-2 system. As the results show below.

• morphological and cultural characteristics

Bacteria strains cultivated in mitis salivaris agar medium were initially distinguished based on their morphological and cultural attributes. The findings indicated that these strains form colonies that are markedly convex, elevated, light-blue, and exhibit a frosted glass-like appearance, with surfaces that can be either rough or smooth. Moreover, these colonies demonstrate a strong adhesion to the agar surface when manipulated with a loop. as shown in figure (1)

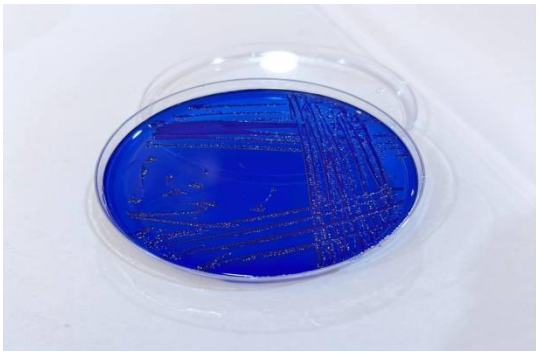


FIGURE 1. - *S. mutans* bacterial isolate streaked on mitis salivaris agar and incubated for 48hours at 37°C.

• Biochemical identification

the biochemical properties of the isolates were examined. The findings indicated that these strains are gram-positive and lack catalase activity. Moreover, they did not demonstrate hemolysin production, instead displaying gamma hemolysis on Blood Agar, as depicted in fig [2]. Based on these outcomes, the isolates were classified as *S. mutans***.**

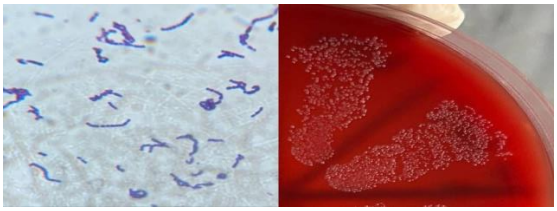


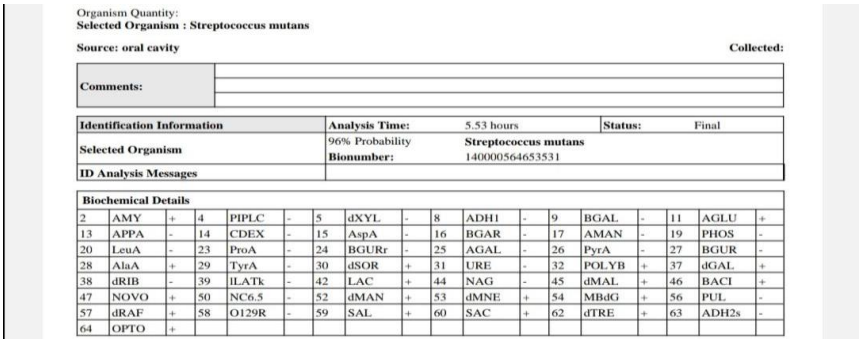
FIGURE 1. *Streptococcus mutans* exhibiting gamma hemolysis on Blood Agar

• Identification by using VITEK-2

The identification of bacterial isolates as *Streptococcus mutans* was confirmed through examination using the automated VITEK-2 system. Results indicated that out of fifty positive samples, only twenty isolates were categorized as *Streptococcus mutans.* The identification of bacterial isolates through automated VITEK-2 revealed the following distribution: 8% *Staphylococcus spp*, 12% *Enterococcus spp*, 6% Lactococcus *spp*, 2% *Pediococcus spp*, 12% other microorganisms such as fungi and yeast, 20% *Streptococcus spp*, and 40% *Streptococcus mutans*. This data is presented in fig (3) and Table (2)

Table 2. the amount of bacteria identification by automated Vitek 2 system.

Type of bacteria	Number of bacteria
Staphylococcus spp	4 (8%)
Enterococcus spp.	6(12%)
Streptococcus spp.	10 (20%)
Streptococcus mutans	20(40%)
Lactococcus spp.	3 (6%)
Pediococcus spp.	1(2%)
Others	6 (12%)



Organism Quantity:
Selected Organism : Streptococcus mutans

Source: oral cavity **Collected:**

Comments:	

Identification Information	**Analysis Time:**	5.53 hours	**Status:**	Final
Selected Organism	96% Probability **Bionumber:**	**Streptococcus mutans** 140000564653531		
ID Analysis Messages				

Biochemical Details

2	AMY	+	4	PIPLC	-	5	dXYL	-	8	ADH1	-	9	BGAL	-	11	AGLU	+
13	APPA	-	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	-
20	LeuA	-	23	ProA	-	24	BGURr	-	25	AGAL	-	26	PyrA	-	27	BGUR	-
28	AlaA	+	29	TyrA	-	30	dSOR	+	31	URE	-	32	POLYB	+	37	dGAL	+
38	dRIB	-	39	ILATk	-	42	LAC	+	44	NAG	-	45	dMAL	+	46	BACI	+
47	NOVO	+	50	NC6.5	-	52	dMAN	+	53	dMNE	+	54	MBdG	+	56	PUL	-
57	dRAF	+	58	O129R	-	59	SAL	+	60	SAC	+	62	dTRE	+	63	ADH2s	-
64	OPTO	+															

FIGURE1. **Results of the identification of *S. mutans* based on their biochemical characteristics were obtained through the utilization of the VITEK-2 identification system.**

3.3 Antibiotic susceptibility of *Streptococcus mutans*

The investigation delved into the antibiotic susceptibility profile of the *S. mutans* strain isolated locally. The findings delineated in Table revealed that *S. mutans* exhibited complete resistance (100%) to Cefepime (FEP). In contrast, varying degrees of resistance and sensitivity were observed towards other antibiotics: 80% resistance to Clindamycin (CD) and Erythromycin (E), 75% resistance to Cefotaxime (CTX), 70% resistance to Penicillin G (P), and 85% resistance to Ceftriaxone (CRO). Interestingly, the strain displayed full sensitivity (100%) to Doxycycline (DXT), Amoxicillin-clavulanic acid (AUG), Trimethoprim-sulfamethoxazole (STX), Vancomycin (VA), Tetracycline (TE), Ampicillin (AMP), and Levofloxacin (LEV). Moreover, it exhibited 90% sensitivity to Meropenem (MRP), as detailed in the table (3) Our results indicate high resistance to Penicillin G, Clindamycin and Erythromycin, This proves it S.*mutans* has multidrug resistance .

Table 3. The susceptibility of the isolated *S. mutans* to different classes of antibiotics.

Antimicrobial agent	P	TE	VA	E	DXT	CD	FEP	SXT	AUG	LEV	AMP	CRO	CTX	MRP
Resistant	70%	0%	0%	80%	0%	80%	100%	0%	0%	0%	0%	85%	75%	10%
Sensitive	30%	100%	100%	20%	100%	20%	0%	100%	100%	100%	100%	15%	25%	90%

3.4 Biofilm Production Assay

The results of biofilm formation to *streptococcus mutans* isolate (20 isolates) .by microtiter plate method indicated that 9(45 %) were strong for biofilms formation, while 7 (35 %) were moderate and 4 (20 %) were demonstrated as a weak biofilm formation,(0%) were reported as non-biofilm producing isolates, in figure (4)

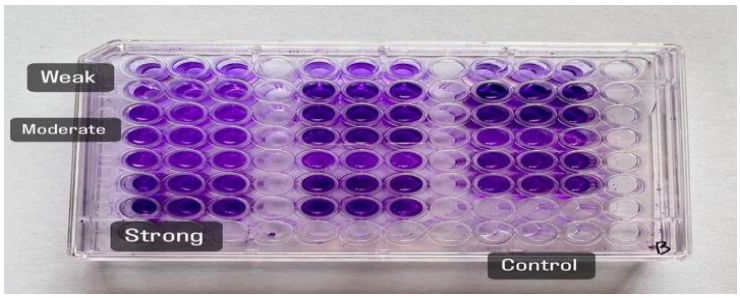


FIGURE1. Microtiter plate test of biofilm positive *streptococcus mutans*

4. DISCUSSION

Dental caries, a prevalent condition in the oral cavity, is primarily instigated by the facultative anaerobic bacterium *Streptococcus mutans*. The formation of biofilm and the facilitation of microbial adhesion to both each other and the tooth enamel are pivotal factors in the etiology of dental caries, potentially progressing to the severe state of infective endocarditis. The prevention of this ailment has persistently posed a significant challenge in dental practice. The principal pathogenic characteristic lies in its capacity to enhance resistance to diverse antimicrobial agents and evade phagocytosis by immune cells (16) In this investigation, specimens collected from the oral cavity of individuals with dental caries were plated on (MSA). The composition of this medium promotes the proliferation of Streptococci while suppressing the growth of alternative bacterial species. Identification of bacterial strains was carried out based on their distinctive colony characteristics observed on MSA medium, which manifested as small, sleek colonies. The findings of this study were consistent with the observations made by Salh and her colleagues (17) in Baghdad City, who reported that cultures of *Streptococcus mutans*. manifested as compact and firmly attached colonies on MSA agar medium. Streptococcus colonies can be readily identified following an extended period of incubation under anaerobic conditions at 37 C. The morphological features observed align with findings reported by Murray *et al* (18). in this study. When subjected to Gram staining, these colonies manifest as Gram-positive cocci, exhibiting a spherical shape and arranged in pairs or short chains. Additionally, they are characterized as non-motile, non-spore forming, consistent with the observations made by James and Natalie (19). Thirty isolates were linked *to Streptococcus spp*., according to the VITEK2 system results, and only twenty isolates (40%) were identified as *Streptococcus mutans*. The findings presented in this study are consistent with the results reported by Israa and Mahdi (20) in AL Kufa city, where it was observed that the prevalence of *S.mutans* in individuals with dental caries was 40 % . A similar observation was made by Abd Al-Zahra and Saleh (21) in a study conducted in Thi Qar province/Iraq, where it was noted that *S.mutans* contributed to 41 % of cases of this particular disease.

Penicillin has been found to reduce infection in prophylactic treatment, but its long-term use may be compromised by resistant strains. Erythromycin and clindamycin are recommended alternatives for patients allergic to penicillin and for endocarditis associated with dental procedures. These antibiotics have developed resistance against *S.mutans*, as shown in this study that result agree with study done by Karikalan, & Mohankumar (22) while disagree with study by Salman & Senthikumar (23) indicate all strain show susceptibility to penicillin and erythromycin . Regarding ceftriaxone and cefotaxime, S.*mutans* isolates exhibited a notable resistance frequency of 85 - 75% towards ceftriaxone and cefotaxime .The research also demonstrated a complete susceptibility of 100% to Levofloxacin , in line with Abobakr, *et al* (24), but contradicting the outcomes of Al-Shami *et al*. (25). This variance could potentially be attributed to the extensive utilization of clindamycin in the management of dental conditions. Notably, strains derived from caries specimens displayed a full susceptibility of 100% to tetracycline and 80% resistance to clindamycin, corroborating the observations of Karikalan *et al*. (26), yet conflicting with the conclusions drawn by Patidar *et al.* (27)

The methodology of utilizing the microtiter plate system, which provides a precise assessment of biofilm development by microorganisms, was implemented in this study to further evaluate the test isolates of *S. mutans* (20) for biofilm formation. Analysis of dental plaque samples revealed their capability to produce significant amounts of robust biofilm structure, as indicated by the microtiter plate results. The current investigation utilized enzyme linked immunosorbent assay [ELISA] to distinguish between *S. mutans* isolates that produced biofilms and those that did not. These biofilms play a role in chronic diseases that pose challenges in terms of treatment, with the median optical density (OD) values at 630 nm being utilized for this purpose. Following the evaluation of twenty different isolates for biofilm development, it was determined that 9 (45%) exhibited high biofilm production, 7 (35%) moderate biofilm production, and 4 (20%) low biofilm production. This finding is consistent with the study conducted by Zayed *et al*. (28). Their research indicated that 35 Streptococcus test isolates obtained from dental plaque (43.75%) demonstrated high biofilm production, while only 26 Streptococcus test isolates obtained from saliva (32.5%) exhibited a similar level of biofilm production. In a similar vein, Abo Bakr *et al* (29) observed that relative to development of biofilm by *S. mutans* alone, 33 % of the isolates produced low level biofilm, 21 % moderate level biofilm and 44 % high level biofilm.

5. CONCLUSION

Locally isolate *S.mutans* was sensitive to different antibiotics , while resisted 100% to Cefepime (FEP) .and it was sensitive to the other antibiotics full sensitivity (100%) to Doxycycline (DXT), Amoxicillin-clavulanic acid (AUG) , Trimethoprim-sulfamethoxazole(STX), Vancomycin (VA), Tetracycline(TE), Ampicillin (AMP), and Levofloxacin(LEV). Moreover, it exhibited 90% sensitivity to Meropenem (MRP),In this study we concluded that the biofilm formation by *S.mutans* clinical isolates play major role in the pathogenicity and also comtribute in the antibiotics resistances .

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