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ISOLATION AND DIAGNOSIS OF STREPTOCOCCUS MUTANS FROM PATIENTS INFECTED WITH DENTAL CARIES

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Abstract: Background: Dental caries, necrosis and gingivitis are among the health problems which Iraqi society suffers from it, as the treatment of bacteria has become a challenge due to their ability to resist many antibiotics. Streptococcus mutans are one of the most common species in the mouth which cause dental caries.

Objectives: The study aimed to isolate and diagnose Streptococcus mutans and study their resistance to a number of antibiotics, and evaluate the susceptibility of Streptococcus mutans isolates to form biofilms, and other Virulence factors such as urease, lipase, Lecithinase, blood hemolysis.

Materials and Methods: Using biochemical tests and phenotypic diagnostics. Using biochemical tests and phenotypic diagnostics. The study included the collection of (155) oral swabs from patients with tooth decay from the area of caries on the surface of the tooth and from cysts for infected patients and visitors at the Al-Fallujah Teaching Hospital with ages ranging between (6-60) years and of both sexes for the period from 1/9/2023 to 1/11/2024.

Results: The number of isolates was 30(19.35%) belonging to Streptococcus mutans, the sensitivity of all the isolates of the bacteria under study was tested towards 11 antibiotics, and and each isolate was selected in order to evaluate its capacity to produce biofilms on a microtitration plate. The findings indicated that the majority of the isolates had a high capacity for biofilm production.

Keywords: Tooth decay, Streptococcus mutans, Antibiotic-resistant, Virulence factors.

Introduction

Periodontal disease is one of the most prevalent diseases in the world such as Dental caries and gingivitis.[1]. The acids present in the oral cavity disintegrate the enamel layer or damage the surface of the tooth root, the concentration of these acids is related to the human diet. [2], as well as these acids cause damage to the supporting tissues of the teeth [3].

Streptococcus mutans is one of the most important causes of dental caries. It is a Gram-positive, facultative anaerobe, as well as have ability to form biofilms, acid production and salt tolerance [4, 5].

tooth caries is a complex, dynamic illness that causes the demineralization and remineralization of tooth hard tissues. It has long been linked to poor oral hygiene and sugar consumption. Caries can destroy the tooth crown and eventually expose the root surfaces of permanent teeth, and it can happen at any point of a tooth's existence. The start and course of dental caries are influenced by the relative abundance of protective and pathogenic substances [6].

Acidogenic byproducts of bacterial metabolism boost the carious potential of the biofilm by causing enamel demineralization and triggering defensive responses in the pulp and dentine, such as increased intratubular dentine and early inflammation. As enamel demineralizes more, bacteria will eventually infiltrate dentine, resulting in further demineralization and ultimately cavitation [7].

The aims of the study include identification of *Streptococcus mutans* and *Streptococcus sobrinus* and study their resistance to antibiotics. Also, their ability formation of biofilms.

Materials and Methods

Sampling and Isolation

The study included the collection of 155 oral swabs from patients with dental caries from the area of caries on the surface of the tooth in Al-Fallujah Teaching Hospital with ages ranging between 6-60 years from both sexes for the period from 1/9/2023 to 1/11/2024. Then the samples were transferred directly to the laboratory to culture it on Mitis Salivarius Bacitracin Agar (MSBA) which contain 200 IU/liter, then incubated in the incubator for 24 hours at 37 C°, then all single colony from plates were sub-cultured to obtain pure culture of the isolates to Identification later.

Identification

In this study the tests (oxidase test, catalase test, urease test, mannitol fermentation test, and Coagulase Production) were conducted to identification the isolates [8, 9], as well as VITEK 2 wase used to certain the identification [10].

Antibiotics Susceptibility

A bacterial sensitivity test for 11 antibiotics (Bioanalyse, Turkey) as mentioned in Table (1) was performed, where all bacterial isolates is studied against antibiotics by Disc Diffusion Method using the Kirby Bauer method [11, 12].

Table 1: Antibiotics and their concentrations

Antibiotics	Code	µg/disk
Tetracyclin	TE	30
Ceftriaxone	CTR	10
Meropenem	MRP	10
Erythromycin	E	15
Gentamycin	GEN	10
Azithromycin	AZM	15
Ciprofloxacin	CIP	10
Imipenem	IPM	10
Augmentin	AMC	30
Vancomycin	VA	30
Cefotaxime	CTX	

Urease test

20% urea was added to urea agar, which was then incubated for 18 to 24 hours at 37 °C. The media's color turning pink was taken to be a sign of a successful outcome [13].

Coagulase production test

The test was conducted by placing two drops of plasma on both sides of the slide, and to one of them added the bacterial growth, while to the other a drop of normal saline was added for the purpose of comparison (control). The occurrence of any clotting of the plasma after (15) seconds compared to the control is evidence of the production of the enzyme. [14]

Lipase and Lecithinase tests

The recipe for egg yolk agar was to combine 5 ml of egg yolk with 95 ml of nutritional agar that had been autoclave-sterilized and allowed to cool to 45 °C. Spooned into sterilized plates was the agar. The bacteria that generate the enzymes lipase or lecithinase were identified using agar [15]. Pure isolated bacteria were used to inoculate egg yolk agar, which was then incubated for 24 to 48 hours at 37 °C. It is assumed that egg yolk agar works well to block the lecthinase enzyme in the vicinity of the growing colonies. An other method for determining the lipase enzyme's efficacy is egg yolk agar. The egg yolk agar test was performed by submerging the dish in an adequate amount of saturated copper sulfate for a duration of 20 minutes. The plate was dried in the incubator for thirty minutes following the removal of extra solution. Growth regions began to become greenish blue, indicating that the lipase enzyme was breaking down fat.

Gelatin liquefaction test

After being aggressively injected with bacterial isolates, a tube of nutritional broth containing 12% gelatin was incubated at 35ºC for five days. After being taken out of the incubator and chilled for at least half an hour, each day was examined. Positive outcome shown by the culture remaining liquid (no gel when chilled) [16].

Hemolysin test

In order to measure the hemolytic activity of bacteria, blood agar containing 5% blood was used. On blood agar plates, pure isolated bacteria were grown and incubated for 24 hours at 37 °C. A positive hemolysin test result is indicated by the formation of a translucent zone surrounding the bacteria [17].

Biofilm formation

Microtiter Plate (MTP) was used for study Biofilm formation, The optical density readings (OD) by using ELISA with a length of 630 nm were [18].

Results and Discussion

Study sample

The results showed that 30 samples (19.35%) of the total samples (155 samples) were positive growth on the selective isolation medium (Mitis Salivarius Bacitracin Agar), while 125 (80.65%) did not show any growth as in the Table (2).

Table (2) percentages of bacterial isolates which isolated from patients

Total (%)	No growth (%)	Growth (%)
155(100)	125 (80,65%)	30(19.35%)

Isolation and Identification

Primary isolation was based on form and color of bacterial colonies on special and selective media. The results showed that the colonies of 30 isolates (19.35%) of the total isolates appeared dark blue with light-colored edges on the Mitis Salivarius Bacitracin Agar (MSBA) which contain 200 IU/liter medium, with a diameter ranging between 1-2 mm as showed in figure (1). The growth of 30 isolates under anaerobic

conditions (5%CO2) was much better then incubating under aerobic conditions. This result matches previous research findings of *Streptococcus mutans [*19,20,21]. Microscopy using the Gram stain shows that all bacterial isolates were cocci form, positive for the Cram stain, bacterial cells arranged in a short chains or pairs as showed in (Figure 6). These results are consistent with previous local and international studies of *Streptococcus mutans* [21,22].

The diagnosis of *Streptococcus mutans* was confirmed by using tests mentioned in the table (3). The results of biochemical test showed that all *S. mutans* isolates were negative for Catalase, oxidase and methyl red tests [23, 24]. As for sugar fermentation tests, the results showed that all isolates of Streptococcus mutans ferment Sucrose, Mannitol and Mannitol. The results of study were conducted in Egypt showed that *S. mutans* isolated from patients with tooth decay had the ability to ferment four main sugars used in their diagnosis: mannitol, sucrose, sorbitol and inulin [25,26]. as well as of S. *mutans* has the ability to ferment and consume lactose, mannose and raffinose, and produce dextran which has an effective role in the pathogenesis of bacteria and caused teeth decay [27, 28]. To confirm the diagnosis of isolates as *S. mutans*, they were also examined by the VITEK2 system.

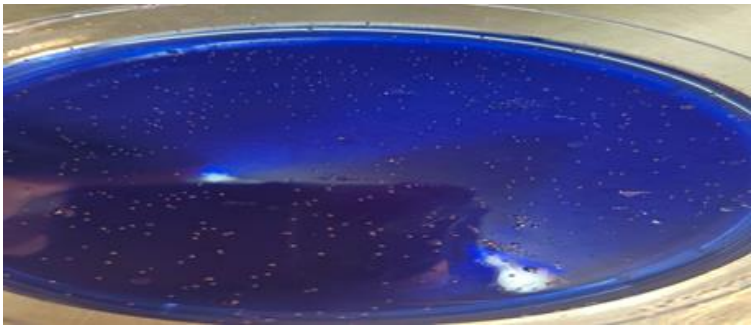


Figure 1: *Streptococcus* mutans on Mitis Salivarius Bacitracin agar

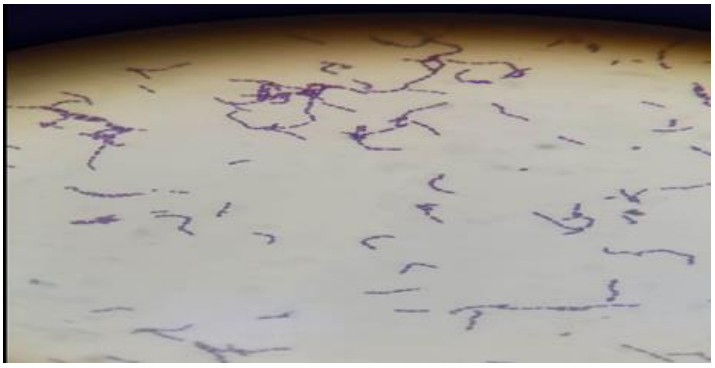


Figure (2) *Streptococcus mutans* under a light microscope (X1000).

Table (3) Biochemical tests of *S. mutans*

Tests	Results
Catalase test	-
Oxidase test	-
Methyl Red	-
Sucrose	+ =
Mannitol	+
Galactose	+

Occurrence of *Streptococcus mutans*

Results current study showed that the percentage of bacteria in females is higher than in males, with 19 (12.25 %) females with *S. mutans* (Table 3). while 11 (7.09%) males were infected with *S. mutans* as in the table (4).

Table (4) Percentage of bacterial isolates which obtained from males and females with dental caries

Total (%)	Negative samples of females (%)	Positive samples of females (%)	Negative samples of (%) males	positive samples of (%) males
155	77(49.67%)	19(12.25%)	48(30.96%)	11(7.09%)

The highest rate of *S. mutans* within the age group 6-10 years, followed by the age group 11-20 years, followed by the age group 31-40 years, followed by the age group 41-50 and 21-30 years, with an isolation rate of 12(40%), 8 (26.67%),5(16.67%), 3(10%), and 2(6.67) respectively as in the table (5). The results of the study were somewhat compatible with local and international studies. The researcher Flayyih *et al*. isolate *Streptococcus mutans* from primary school students and from patients with private clinics auditors in Baghdad were 15 isolates of the totals of 109 patients with a percentage (13.79%) using the different selective and rich media to isolate *Streptococcus mutans* The highest isolation rate for females was compared to males within different age groups, which was (49.3 %) in the age group of females (4-12) years, and (39.3 %) in the age group of males (4-12) years [29,30] .

Table (5) Distribution of *Streptococcus mutans* bacteria by age groups in patients with dental caries

Age group	Isolates of *Strep. mutans* in females	Isolates of *Strep. mutans* in males	Total (%)
10-6	21 (36.84%)	5(45.45%)	12(40%)
20-11	5(26.32%)	3(27.27%)	8(26.67%)
30-21	0(0%)	2(18.18%)	2 (6.67%)
40-31	4(21.05%)	1(9.09%)	5(16.67%)
50-41	3(15.79%)	0(0%)	3(10%)
60-51	0(0%)	0(0%)	0(0%)
Total (%)	19(100)	11 (100%)	30(100)

Virulence factors

Blood hemolysis

Hemolysin test was used to investigate the production of blood enzyme. The results of blood agar hemolysis showed some *S. mutans* has the ability to hemolysis the blood (alpha type) with percentage 15%, while others strains could not hemolysis the blood (alpha type), this is evidence of the ability of bacteria to produce hemolysin, as for other isolates of *S. mutans* could not hemolysis the blood (gamma type) with percentage 15%, as in figure (3), these results were agreed with what was stated in previous studies [31].

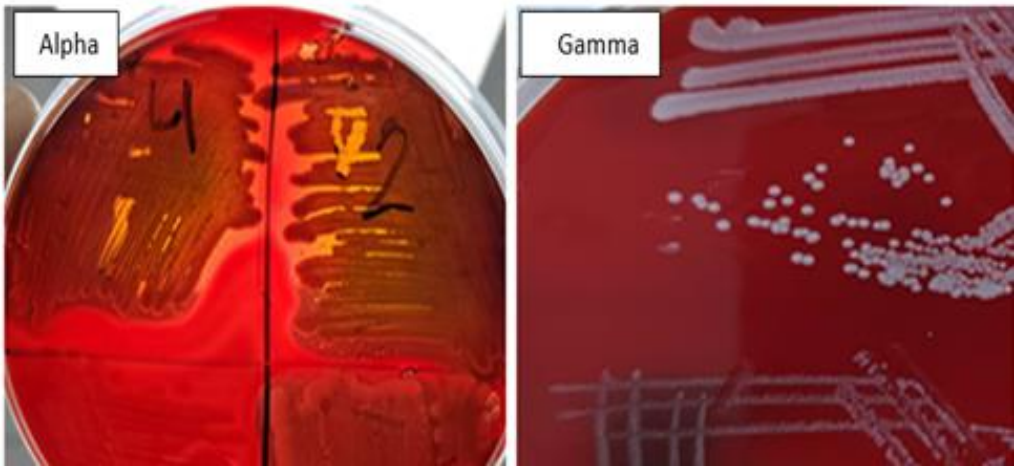


Figure (3) Colonies of *S. mutans* on blood agar

Urease production

This experiment was conducted to examine the urease production capacity of bacteria and to assess urea levels. The outcomes demonstrated that, as indicated in the table (6), none of the isolates could manufacture the urease enzyme.

Lipase and Lecithinase

The results showed all isolates were unable to produce Lipase and Lecithinase as in the table (6).

Coagulase

The results of this study appeared all isolates of *Streptococcus mutans* were unable to produce Coagulase enzyme as in the table (6).

Table (6) Virulence factors of *Streptococcus mutans*

Tests	Results
Blood hemolysis	Gamma, Alpha
Urease	-
Lipase	-
Lecithinase	-
Coagulase	-

Biofilm formation

Bacterial biofilm formation susceptibility is shown in figure (5), where it is observed that 5 isolates (9) have a strong biofilm-forming capacity, while isolates (15) have a moderate ability to build membranes and isolates (6) have a poor biofilm-forming ability. Based on the researchers' study of a microplate plate, Table (5) revealed that 30 (100%) of the bacterial isolates had the capacity to build membranes at different speeds [32,33].

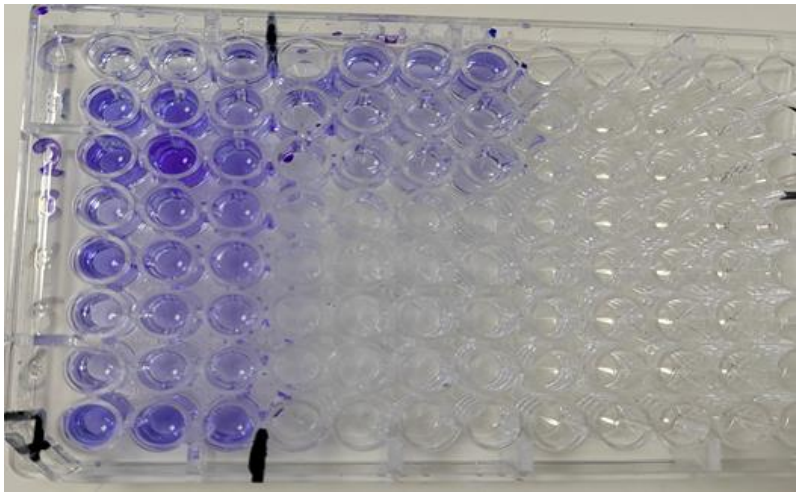


Figure 5: The capacity of S. mutans isolates (1, 2, 4, 7, 8) to form biofilms is high, that of isolates (3, 5, 6) to create membranes is medium, and that of isolates (10, 9) to produce membranes is poor. Positive Control: C+, Negative Control: C-

Table (9): Number of *S. mutans* and the percentages of its ability to form biofilms

Number of isolates (N)	Percentage (%)	Biofilms Formation
15	50	Strong
9	30	Moderate
6	20	Weak
0	0	No Biofilm
10	100	Total

The bacterial sensitivity test for antibiotics was performed using Muller-Hinton Agar and 11 types of antibiotics. The current study showed that bacterial isolates show a difference in their resistance and sensitivity to the antibiotics under study. Fig. 10 and Appendix 3 showed that the Strep.mutans, the results showed that (27) isolates have high resistance to Tetracycline, Erythromycin by (90%), followed by Cefotaxime (24) isolates have resistance (80%) followed by Azithromycin have resistance 21(70%), Figure (9) Table (7). While bacterial isolates recorded the highest percentage of sensitivity to Gentamycin 30 isolates have sensitivity (100%)followed by Augmentin (80%),followed by Imipenem (70%), followed by Ceftriaxone (60%),followed byVancomycin,Meropenem (50%)

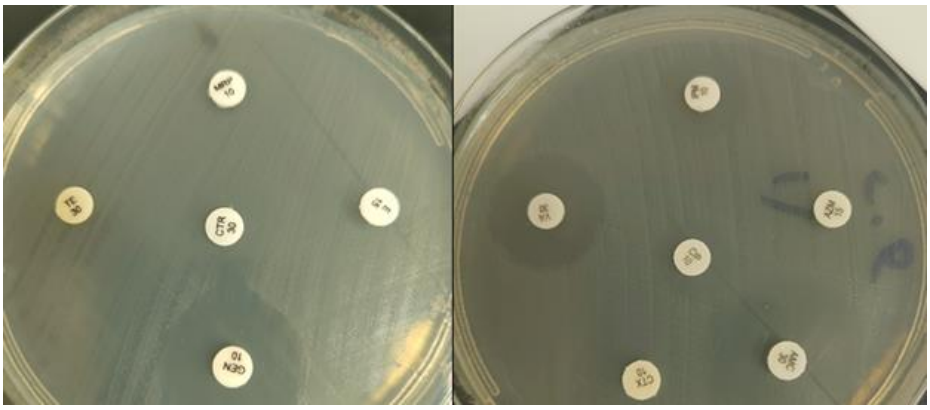


Figure (4) Resistance and sensitivity of some isolates of S. mutans and to a number of antibiotics

Table (7) Susceptibility of (6) isolates of *S. mutans* to antibiotics

Antibiotics	Cod	Resistant isolates (R)		Intermediate isolates (I)		Sensitive isolates (S)	
	AK	Number	Percentage%	Number	Percentage%	Number	Percentage%
Tetracyclin	TE	27	90%	0		3	10%
Ceftriaxone	CTR	12	40%	0		18	60%
Meropenem	CTR	15	50%	0		15	50%
Erythromycin	E	27	90%	0		3	10%
Gentamycin	GEN	0	0	0		30	100%
Azithromycin	AZM	21	70%	0		9	90%
Ciprofloxacin	CIP	15	50%	0		15	50%
Imipenem	IPM	3	10%	6	20%	21	70%
Augmentin	AMC	6	20%	0		24	80%
Vancomycin	VA	15	50%	0		15	50%
Cefotaxime	CTX	24	80%	3	10%	3	10%

*R: Resistance, I: Intermediate, S: Sensitive

It was found that the S. mutans isolates were susceptible to several of the antibiotics being investigated, and these findings are consistent with those of a few previous investigations conducted by other researchers. The new study's findings are in line with earlier research, particularly the conclusions made locally by [33,34]. One of the main reasons for bacterial resistance to antibiotics is the horizontal transfer of genes, as it is the reason for the development of the ability of bacteria to resist pesticides and antibiotics through the mechanism of gene transfer from bacteria to other bacteria through plasmids or through viruses known as phages or through sexual filaments [35,36].

Conclusion

Conclusion The results of the current study showed that the percentage of isolation of S. mutans isolated from the mouth 19.35%. and Isolates are characterized by their antibiotic constituent (Tetracyclin, Erythromycin. Azithromycin, Cefotaxime, Meropenem), and less resistant (Ceftriaxone, Augmentin and Imipenem). And The current study showed that all isolates had the ability to form biofilms differently, where 50% had the ability to form strong biofilms and 30% formed medium-strength biofilms and 20% formed Weak -strength biofilms.

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