

Isolation and Identification of Oral Bacteria and Characterization for biofilm formation and antibiotics resistance

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ABSTRACT: In this study, 100 bacterial isolates were obtained from tooth surface swabs of male and female patients with different ages who visited specialized centers and dental clinics in Baghdad city after they were clinically diagnosed with oral (dental) infections by specialist doctors for the period from 1/10/2022 to 1/3/2023.

The methods employed to identify the bacterial isolates were the Vitek system, as well as highest isolated rate was among *Streptococcus mutans* species (48%). Isolates of *S. mutans* and their drug sensitivity was tested against 10 antibiotics using the disc diffusion process, showing that the isolates' Antibiotic resistance was larger than their sensitivity to these antibiotics. The resistance rate of the isolates to erythromycin was (91.12%) before biofilm formation and (98.12%) after biofilm formation, the resistance rate of the isolates to amoxicillin was (45%) before biofilm formation and (50%) after biofilm formation, to ampicillin was (95%) before biofilm formation and (100%) after biofilm formation, to Nalidixic acid (44.3%) before biofilm formation and (80.35%) after biofilm formation, to cefotaxime the resistance rate was (62.64%) before biofilm formation and (69.64%) after biofilm formation, and to tetracycline the resistance rate was (50.35%) before biofilm formation and (55.35%) after biofilm formation, while the isolates showed high sensitivity to the antibiotic amikacin (75%) before and after biofilm formation.

Keywords: *Streptococcus*, antibiotics, biofilm



1. INTRODUCTION

Streptococcus mutans is a member of Microorganisms with Gram-positive bacteria and is one the most prominent biofilm producing bacterial species. It colonizes the mouth during the first days after birth, and over time, other Gram-positive and Gram-negative species accompany the streptococci [1].

A biofilm represents a bacterial population whose cells are attached to a semi-submerged surface and are mainly covered with a matrix of extracellular polymeric substances (EPS) resulting from cells that exhibit a different cellular morphology when they become free in terms of growth rate and gene expression [2]. The presence of biofilm in the human body causes a number of diseases, including cystic fibrosis, endocarditis, otitis media, prostatitis, periodontitis, dental decay, etc [3]. It is also believed that the interaction between the bacterial species in the mouth during the biofilm formation leads to a clash in the mechanics of bacterial interaction through the signals they release to form the biofilm. From this standpoint, it is possible to imagine the interaction between the biofilm-producing bacterial communities as war and peace [4].

The repeated and increasing use of antibiotics in treating medical conditions for long periods of time has led to the emergence of side effects that harm the individual's health on the one hand and the emergence of antibiotic-

resistant strains on the other hand [5]. Therefore, thinking began about using or finding other alternatives represented by changing the treatment strategy in terms of regulating the presence of Commensal microbiota that affect the stability and organization of the oral environment through their antibacterial effect, and because of the resistance of the biofilm to antibiotics, the host's immune system, and the organism's physiological characteristics, in addition to their possession of resistance genes, therefore, it was necessary to study the virulence factors of these germs from a genetic perspective, which leads to getting closer to reducing the effect of these germs on human teeth. In addition, some of the characteristics of the virulence factors of the pathogenic *S. mutans* bacteria can lead not only to knowing or understanding the role of these factors in the development of caries, but also it can lead to getting closer to increasing the reduction of the effects of such factors on caries [6]. The interaction between bacterial species plays a role in the dynamics of bacterial communities. Some of them promote the formation of tooth blackening and the development of other diseases, including tooth decay and periodontitis. Thus, it is better to understand the interaction between oral pathogenic and commensal bacterial cells and the effect of this interaction on the gene expression of some virulence and pathogenicity factors. It is believed that the presence of types of bacterial flora can lead to the difference in the effect on biofilm formation and gene expression of virulence factors of *S. mutans* [7]. **The aim the this study** Isolate and diagnose *Streptococcus mutans* bacteria from dental caries, because they are the most common pathogenic causes of dental caries. Study the ability of these bacteria to form biofilms and Test the ability of *Streptococcus mutans* bacteria to resist antibiotics.

2. MATERIALS AND METHODS

2.1 Sample collection

In this study, 100 bacterial isolates were collected from patients of both sexes and of different ages with dental decay who visited specialized centers and dental clinics in Baghdad city in a period of five months from (1/10/2022) to (1/3/2023). The samples were taken by sterile bonding brushes, and the swabs were placed in tubes containing transport media and sent to the laboratory to be cultured on the appropriate nutrient media and conduct diagnostic tests.

2.2 Transport media

The brain heart infusion broth media (B.H.I.B) was used for transferring swabs to the laboratory and then grown on the appropriate nutrient media until diagnosis

2.3 Bacterial isolation and identification

A- Isolation

Samples of cotton swabs taken from oral infections were grown and incubated for a full day at 37°C. for the purpose of activating the bacteria using the streaking method on enriched culture media, such as blood agar and MacConkey agar, both aerobically and anaerobically. The plates were incubated for 24 hours at 37°C. After incubation, the morphologic and biochemical tests were performed.

B- Identification by using Vitek-2 compact system

The Vitek system is one of the modern and rapid diagnostic systems for bacterial diagnosis, as it gives up to 99% accuracy results. To confirm bacterial isolates, this system was used according to the manufacturer's instructions according to [8]:

- 1- Bacterial isolates were cultures on blood agar and MacConkey agar and incubated at 37°C for 24 hours.
- 2-A bacterial suspension was prepared from the bacterial culture, by transferring one colony from each dish to test tubes containing 3 ml of 0.85% normal saline solution, then The expansion turbidity was decreased in order to receive a

suspension with a density ranging employing a spectrophotometer with a 230 nm wavelength to measure between 0.50 and 0.63 mg. ml⁻¹, or 1.5 x 10⁸ cells. the solution.3- After inserting the card cassette for bacterial species diagnosis into each test tube holding the tubes were placed in the diluted bacterial suspension in the Vitek system, which automatically reads the results and determines the bacterial species in the suspension.

2.4 Preservation and maintenance of bacterial isolates

- 1- Short-term storage: Bacterial isolates were maintained for a few weeks on MacConkey agar. The culture medium was poured as a slant, the plates were inoculated, then wrapped well with parafilm and stored at 4°C [9].
- 2- Long-term storage: The brain and heart infusion medium was used by adding 20% glycerin. The medium was distributed in small, tightly sealed bottles with a volume of 10 ml for each, then sterilized with an autoclave and then inoculated with 0.01 ml of *Streptococcus spp* bacteria cultures. They were then incubated at 37°C for 24 hours and then stored at -20°C [10]. This method was used to preserve bacterial cultures for a long period of up to three months.

2.5 Biofilm formation

The method of Christensen et al; (1985), called the tissue culture plate method, was adopted to determine biofilm formation, which is summarized in the following steps:

- In the case of biofilm formation by *S. mutans* bacteria, we culture them on T.S.B medium. contained 1% glucose, was incubated at 37°C for 18 hours, and was subsequently diluted. to 1:100 with glucose-free T.S.B medium.
- A sterile plate containing 30 wells is prepared, and the bacterial culture containing only *S. mutans* bacteria is placed inside the wells, using three duplicates per rate. Additionally, the T.S.B. liquid glucose-free medium is placed as a control for a number of wells.
- At 37°C, the plate is incubated for 24 hours., then all the well contents are carefully removed by inverting the plate, and then Phosphate Buffer Saline (PBS) solution (pH=7.2) is used to wash the wells four times.), to get rid of the solution floating with non-adherent bacteria.
- The plate is incubated for 24 hours at 37°C, then all contents of the pits are carefully removed by turning the plate over, and then the pits are using Phosphate Buffer Salin for four washing cycles (PBS) solution, pH = 7.2, to get rid of the floating solution with non-adherent bacteria.
- The biofilm is formed by the bacterial adherence to the plate and is adjusted by placing it in the incubator at 37°C for 30 minutes.
- The wells are stained with 1% crystal violet (w/v), then washed with deionized distilled water, then left to dry.
- 150 microliters acetone:ethanol (20:80) (v/v) is added to the wells, then the plate is placed in the Eliza reader to read the absorbance, writing and analyzing the results In light of the table 1:

Table 1. Adhesion and formation bacterial biofilm through the TCP method according to (12)

| Biofilm formatic | Adherence | The average OD value at 630 nm |
|------------------|-----------|--------------------------------|
| Non | Non | 0.120< |
| Moderate | Moderate | 0.120 – 0.240 |
| High | High | 0.240> |

2.6 Investigating the drug sensitivity of *S. mutans* isolates to some antibiotics

S. mutans bacteria was cultured (grown) in (T.S.B.) medium to measure their sensitivity to antibiotics, then they were grown on (T.S.B.) medium containing 1% glucose to examine their sensitivity to antibiotics after the biofilm formation. Drug susceptibility of bacterial isolates was tested by the disc method according to the method of [11] in the following steps:

- 1- The cotton swab was dipped in the soybean tryptone broth and the excess was removed by pressing it against the inner sides of the tube.
- 2- The bacterial isolates were spread on Mueller-Hinton agar by streaking more than twice and in different directions to ensure that the bacterial isolates whose sensitivity was to be tested were spread evenly, and the plates were left for 15 minutes at room temperature to ensure moisture absorption.
- 3- The antibiotic discs (5 discs) were placed in a 100 mm plate, and (12 discs) were placed in a 150 mm plate, and the distance between each disc and the other was 24 mm (from the center of the first disc to the center of the other disc).
- 4- The plates were incubated at 35°C for (16-18) hours for all types of antibiotics, then Measurements were made of the inhibition diameters and compared with the standard values mentioned in [12].

3. RESULTS AND DISCUSSION

First: Isolation and identification of bacteria colonizing the mouth

In this study, 100 swabs were collected from people suffering from mouth and gum infections and tooth caries, and the bacterial and fungal species were obtained, which were identified as Gram-positive bacteria using the Vitek system. The results in Table (2) showed the dominance of *S. Mutans*, which recorded the highest rate 48% of the total bacteria isolated from tooth caries, followed by *Staph epidermidis* 22%, then by *Staph aureus* 18%. *Candida* yeast also recorded 12% isolation rate. The dominance of *Streptococcus* bacteria over the rest of the bacteria isolated the results of the current investigation are in accordance with [13] as it is the main cause of caries lesions. Also, the dominance of the *S. mutans* species over the rest of the other species is consistent with the finding of [14] regarding the dominance of the *S. mutans* bacteria over the rest of the bacteria that cause tooth caries. Also, the appearance of *Staphylococcus* bacteria, especially *Staph epidermidis* in the mouth vicinity agrees with the result of [15] who stated that the cause of this bacterium's oral dissemination vicinity may be due to its being one of the important pathogens with the ability to cause opportunistic infections due to its natural presence on the bodies of carriers, on the skin and on the upper part of the nose, digestive and reproductive tracts, or because it possesses many surface antigens and enzymes that help it penetrate the body's tissues [16]. The dominance of *Candida* yeast in the mouth over other types of yeast is consistent with [17], as fungal infections in immunocompromised patients always include *Candida* yeast, due to their ability to show their pathogenicity as true pathogens in some circumstances, such as their production of the enzymes Proteinase, Uronidase, Collaginase, and Neuroaminidase, which help them penetrate the tissues and cells of the host and cause diseases [18].

Table 2. Bacteria isolated from the mouth and their percentages

| Isolates | Number | % |
|---------------------------|--------|----|
| <i>S. mutans</i> | 48 | 48 |
| <i>Staph. epidermidis</i> | 22 | 22 |
| <i>Staph. aureus</i> | 18 | 18 |

| | | |
|------------------|-----|-----|
| C. tropicalis | 12 | 12 |
| Total | 100 | 100 |

3.1 Biofilm formation by *S. mutans* bacteria

formation of biofilms in a micro titer plate was tested on the liquid soybean tryptone medium plus 1% glucose, and according to the absorbance degree (Optical density) (OD) at a 630 nm wave length, the results shown in table (1) were as follows:

Biofilm formation is high when the absorbance is greater than 0.240 nm and moderate when it is between (0.120-0.240) nm. It was shown that 27(56.2%) out of 48 isolates showed high biofilm formation and 15(31.2%) isolates showed moderate biofilm formation, while 6(12.5%) isolates did not show any adhesion or biofilm formation i.e. (at less than 0.120 nm absorbance), and these results were consistent with the results of [19 and 20]. These results showed that biofilm-forming isolates have a high tolerance to acidity levels in dental caries areas, in addition to their ability to reduce oral pH due to their consumption of sugars (glucose, fructose and lactose) and their production of lactic acid as a final product that causes mouth acidity. This is expected because these bacteria possess two properties, acidogenesis and acidouricity [21] In addition, the reason why some *S. mutans* isolates did not form a biofilm may be because the oral swabs were taken from people on antibiotic treatment or using a type of mouthwash that reduces virulence factors of *S. mutans*.

Table 3. Percentages of biofilm-forming *S. mutans* isolates

| Bacteria | High biofilm formation | | Medium composition biofilm composition | | No biofilm formation | |
|------------------|------------------------|------|---|------|-----------------------|------|
| <i>S. mutans</i> | Number of isolates | % | Number of isolates | % | Number of isolates | % |
| | 27 | 56.2 | 15 | 31.2 | 6 | 12.5 |

3.2 Resistance of *S. mutans* isolates to antibiotics

A drug sensitivity test was carried out for all *S. mutans* isolates included in the study, which cause tooth caries (being the first, most frequent and common cause) to a group of antibiotics and their resistance to them by measuring the diameter of the growth inhibition zone around the antibiotic discs used and comparing them with what was mentioned by [22].

Table (4) Figer(1) and Tabl (5) Figer(2) showed that all *S. mutans* isolates that cause tooth caries showed 95% resistance to the antibiotic Ampicillin before biofilm formation, and showed 100% resistance after biofilm formation. While the isolate showed 94% resistance to the aminoglycoside (Streptomycin) before biofilm formation, and 100% resistance after biofilm formation. Also, the isolate showed 91.12% resistance to Erythromycine before biofilm formation and 98.12% resistance after biofilm formation. The isolate also showed 62.64% resistance to Cefotaxime before biofilm formation, and 69.64% resistance after biofilm formation, and showed 54.14% resistance to Gentamycine before biofilm formation and 57.14% resistance after biofilm formation. The *S. mutans* isolates showed 50.35% resistance to Tetracycline before biofilm formation and 55.35% resistance after biofilm formation. As for Amoxicillin, the resistance rate for all bacterial isolates varied from 45% before biofilm formation to 50% after biofilm formation. Among the Macrolides group, the isolates showed varying and slight resistance to the antibiotic Azithromycin ranging from 30.3% before biofilm formation to 33.3% after biofilm formation. From the Lincomsides group, the isolates showed 30.3% resistance to the antibiotic Clindamycin before biofilm formation and 33.3% after

biofilm formation, while the isolates showed 20% to the antibiotic amikacine before biofilm formation and 25% after biofilm formation, which was less resistance rate.

It is also observed from the two tables that the resistance of the isolates to antibiotics increased after biofilm formation, which indicates an increase in their virulence after biofilm formation by these isolates. This is due to the composition of the biofilm and the physiological characteristics of its constituents, which control the rates of antibiotic molecule entry to the membrane through the extracellular carbohydrate molecules that make up it, which works to hinder the transfer of antibiotics into the membrane or through their ionic association with other antibiotic molecules [23]. Another mechanism acquired by the accumulated biofilm-forming bacterial colonies is the slow ability to take up antibiotics by the bacteria located in the deep lower layer of the biofilm due to the lack of access of oxygen and nutrients to them, which is limited to the upper layer of the biofilm that is affected by the antibiotic only (3). Also, the high density that results from the increase in the contact characteristic of the biofilm-forming bacterial cells with each other leads them to acquire a state of heterogeneity, which is considered one of the important causes of antibiotic resistance through plasmids carrying resistance genes among them [24]. In addition, because the biofilm remains for a long time and some of its upper layers (called permanent cells) assume a state of quiescence upon continuous exposure to antibiotics. Even if they are in high concentrations with a killing effect, these bacteria possess phenotypic characteristics that differ from the rest of the other bacteria that make up the upper layer of the biofilm. Thus, the bacteria acquire high antibiotic resistance and replace the damaged bacteria that were killed with other more resistant and more virulent bacteria [25].

Resistance of *S. mutans* to macrolides, including azithromycin, is common. Resistance arises through the production of the RNA methylase enzyme encoded by the *erm* gene [26] and azithromycin is included in the list of antibiotics that are highly resisted by *S. mutans*, where the resistance rate in some strains has reached 100% [27], which is close to the resistance rate of macrolide compounds in the current study.

In addition, the marked variation shown by *S. mutans* isolates in their resistance to antibiotics used may be due to the diversity in resistance mechanisms through the production of beta-lactam enzymes that inhibit these antibiotics or through changing the binding sites of penicillin binding proteins (PBPs) [20]. The other reason depends on the virulence factors of the bacteria itself; as some isolates show more resistance than others, and the difference in testing conditions and the type of techniques used in the study. These reasons, all together create a difference in resistance levels [28].

It was confirmed by (14) that resistance to penicillin's does not only include the production of penicillin and beta-lactam enzymes, but also extends to the production of penicillin-binding proteins (PBPs) located in the cytoplasmic membrane bound to the cell wall. These proteins have enzymatic activity such as Transpeptidases and Carboxypeptidases, as these proteins are targets for both (penicillins and cephalosporins), they work to change the target of anti-beta-lactams and thus produce bacterial resistance to these antibiotics. Most of the resistance shown by *S. mutans* strains is of plasmid origin, because plasmids play a role in the production of beta-lactam enzymes and are responsible for encoding some penicillin-binding proteins (PBPs) [29]

The resistance of our isolates to nalidixic acid occurs after exposure to this antibiotic through chromosomal mutations that lead to a change in the target site represented by the enzyme DNA gyrase, which works to coil the DNA strand (Poirel et al., 2006). Resistance to chloramphenicol is also caused by the drug destruction by *S. mutans* isolates by the enzyme Chloramphenicol acetyl transferase [30]. The mechanism of resistance to Amoxicillin, which is one of the antibiotics with a synergistic effect and is characterized by its resistance to beta-lactam enzymes, does

not differ from the mechanisms of resistance to other antibiotics by changing the target site of the anti-beta-lactam or by reducing the permeability of the antibiotic through the bacterial cell membrane [31].

1. The reason for the resistance shown by bacterial isolates to gentamycin and tetracycline is attributed to their topical use or to the occurrence of a mutation that affects the osmosis of the outer membrane of this antibiotic [32].

Table 4. Percentages of sensitivity of isolates under study to antibiotics before biofilm formation

| Antibiotic | S. mutans | |
|-----------------|-----------|-------|
| | R | S |
| Ampicillin | 95 | 0 |
| Amoxicillin | 45 | 50 |
| Streptomycin | 94 | 0 |
| Chloramphenicol | 33.3 | 66.7 |
| Nalidixic acid | 44.3 | 66.7 |
| Tetracycline | 50.35 | 44.65 |
| Gentamycine | 54.14 | 42.86 |
| Cefotaxime | 62.64 | 30.36 |
| Amikacine | 20 | 75 |
| Erythromycine | 91.12 | 1.88 |

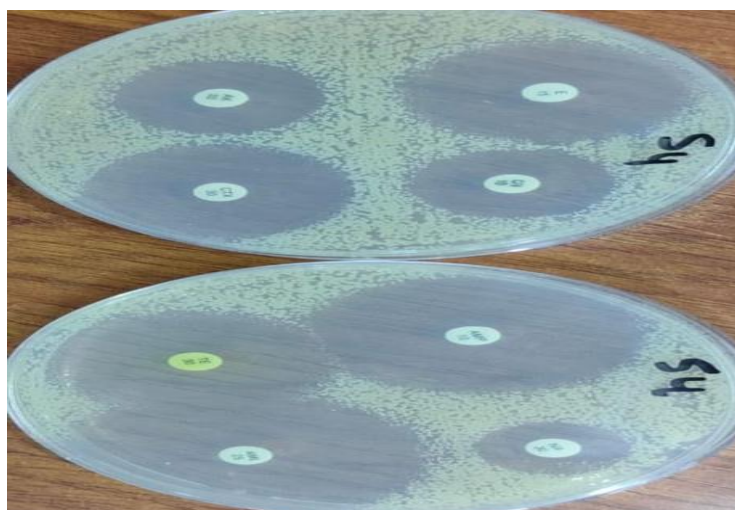


FIGURE 1. Percentages of sensitivity of isolates under study to antibiotics before biofilm formation

Table 5. Percentages of sensitivity of isolates under study to antibiotics after biofilm formation

| Antibiotic | S. mutans | |
|----------------|-----------|-------|
| | R | S |
| Ampicillin | 100 | 0 |
| Amoxicillin | 50 | 50 |
| Nalidixic acid | 80.35 | 66.7 |
| Tetracycline | 55.35 | 44.65 |
| Gentamycine | 57.14 | 42.86 |
| Cefotaxime | 69.64 | 30.36 |
| Amikacine | 25 | 75 |
| Erythromycine | 98.12 | 1.88 |

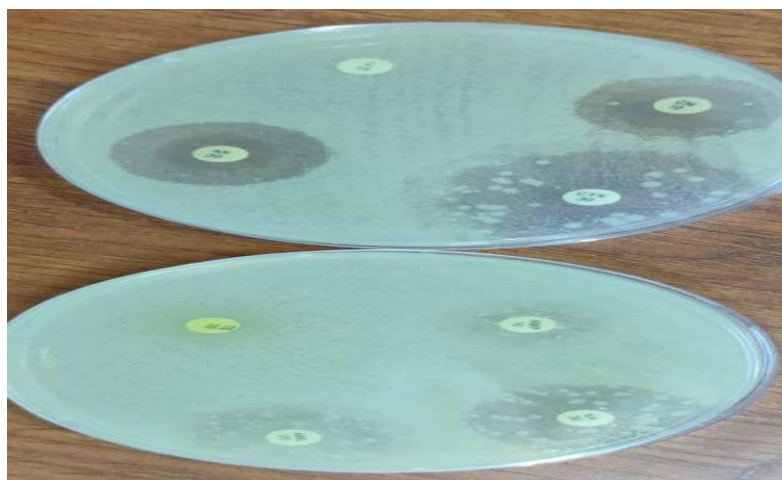


FIGURE 2. Percentages of sensitivity of isolates under study to antibiotics after biofilm formation

4. CONCLUSION

1. *S. mutans* bacteria's dominance as a primary cause of oral infections in humans, especially tooth caries, which plays a major role in increasing the spread of bacteria in the mouth and their formation of biofilms.
2. The capacity of *S. mutans* isolates to show multiple resistance to a number of studied antibiotics because they possess genes for virulence factors and biofilm formation.

5. RECOMMENDATIONS

1. Conduct a periodic examination of the mouth to detect pathogenic agents to avoid the occurrence of asymptomatic infection, which could develop into symptomatic infection that affects the mouth condition.
2. Refrain from using antibiotics continuously and randomly because of their negative impact on the bacterial diversity balance within the body in general and the mouth in particular.

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