

ISOLATION AND IDENTIFICATION OF MUTAN'S STREPTOCOCCI BACTERIA FROM HUMAN DENTAL PLAQUE SAMPLES

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Abstract

Fifty plaque samples were collected from teeth. Forty five samples were considered to be positive bacterial isolates about (10^4 bacteria/ml) using selective Ms-agar (Mitis-Salivarius agar) medium. Thirty isolates were considered to be related to the genus *Streptococcus* and specially to the mutans streptococci of various group; *S. sobrinus* (serotype D, G), *S. mutans* (serotype C, F), *S. cricetus* (serotype A) and *S. rattus* (serotype B) with percentages of (39.29%), (30.30%), (18.18%) and (3.03%), respectively depending on biochemical and Lancefield grouping identification systems.

Key words: Mutan's streptococci bacteria ; human dental plaque; Lancefield grouping identification systems.

Introduction

Dental plaque is an adherent deposit of bacteria and their products, which forms as a white greenish or even yellow film on all tooth surfaces [1]. Dental plaque accumulates naturally at stagnant or retentive sites formed after one to two days with no oral hygiene [2].

Dental caries (tooth decay) have plagued human since the dawn of civilization and still constitutes one of the most common human infectious disease in different parts of the world [3].

Mutans streptococci (*Streptococcus mutans* and *Streptococcus sobrinus*) are the most important bacteria in the pathogenesis of dental caries due to many epidemiological, experimental and animal studies [4,5]. This is due to their ability of rapid lactic acid formation from dietary carbohydrates, mainly sucrose and glucose [6].

Principle identification or diagnosis of mutans streptococci is usually made from the characteristic morphology of its colonies on 5% sucrose containing culture media. Mitis-Salivarius medium, is usually used, which is composed of mitis salivarius agar with sucrose and potassium tellurite. It has the ability to inhibit growth of most bacteria, except streptococci, because it contains trypan blue and crystal violet, which suppress the growth

of gram-negative organisms [7]. MS-agar is a logically starting point for the development of further selective culture media. Van Houte [8] modified MS-agar by adding 0.2 U/ml bacitracin and increasing the sucrose concentration to 20%, to inhibit growth of *Streptococcus sobrinus* and *Streptococcus cricetus*. This medium is called "Mitis-Salivarius bacitracin" (MSB) agar [9].

Further identification of mutans streptococci after culturing on the selective and non-selective agar media is, gram-staining, distinctive cell shape on light microscopy and biochemical test (including antibiotic-sensitivity test, the ability to tolerate 4% NaCl, the ability to produce dextran or levans, and sugar fermentation and enzymatic patterns). Mutans streptococci can also be further identified by the use of a commercial biochemical test system like API 20 Strep and the Lancefield grouping kit [10]. The aims of the study isolation and characterization of mutans streptococci.

Materials and Methods

Collection of Samples

Fifty plaque samples were collected from staff of Biotechnology Department in College of Science, Al-Nahrain University, in sterilized tubes containing 2ml normal saline. Samples

were stored in a cool place then transported to the laboratory.

Isolation of Mutans Streptococci Bacteria

One hundred microliter of undiluted samples were spread on the surface of MS-agar plates using sterile swabs. Cultures were incubated anaerobically for 48 hrs at 37°C and aerobically overnight at 37°C. Count of more than 250 colonies (10^4 cells/ml) was considered as positive samples [10].

Identification of Isolates

After estimation of positive samples on the surface of MS-agar medium, small colonies were subcultured on the surface of blood-agar plates for further purification and incubated anaerobically for two days at 37°C.

The following methods were used for initial characterization of the isolates:

- a. Colonial shape and form on MS-agar and blood agar.
- b. Gram-staining and microscopic examination.
- c. Catalase test.
- d. Dextrane production test.

Dextran production test was done following the method of [11].

Rapid Differentiation of Colonies

A differentiation between colonies belongs to mutans streptococci from that of other streptococci like *S. sanguis* on MS-agar could be achieved by the inoculation of the MS-agar with the tested isolate and incubated anaerobically at 37°C for two days. Then a test solution of (10%) mannitol and (4%) of 2,3,5-triphenyltetrazolium chloride (TTC) were spread on the plates.

A change in color to a dark pink was developed due to hydrolysis of mannitol to acid by the enzyme mannitol-1-phosphate dehydrogenase and a reduction of (TTC) which could taken as an indicator for the presence of mutans streptococci [12,9].

Tolerance to High Concentration of Sodium Chloride

The susceptibility of bacterial isolates to (4%) NaCl was tested using TYS-broth medium incubated anaerobically at 37 °C for 48 hrs. Growth was monitored visually as compared with a control tube which did not

contain this concentration of sodium chloride. Turbidity is an indicator for the ability of the bacterial isolates to tolerate this concentration of NaCl.

Utilization of Different Carbohydrates Sources

The ability of the bacterial isolates to utilize different carbohydrate sources was determined following the method described by [14].

Brain heart infusion broth supplemented with (10%) of each carbohydrate (sucrose, mannitol, sorbitol and Inulin). Sucrose was used as positive control and brain heart infusion broth medium as negative control, carbohydrates solutions were sterilized by filtration and added aseptically to the autoclaved brain heart infusion broth medium which contains a (0.02%) of phenol red, then, the suspended media was inoculated with the tested isolates and incubated anaerobically at 37°C for 72 hrs. The change in the color of media from red to yellow as compared with the negative and positive control indicated the ability of these tested isolates to utilize these carbohydrates source. For further conformation bacterial growth was measured using spectrophotometer and pH change was also measured using pH meter.

Antibiotic Sensitivity Test

Disk diffusion method for antibiotic sensitivity test was determined following the method described by Baron *et al.* [15]. Brain heart infusion broth (10 ml) was inoculated with a loopfull of bacterial isolate, the culture was incubated at 37°C to mid log phase. A (0.1 ml) of inoculated broth transferred to Muller-Hinton agar plates.

A sterile cotton swab was used to streak the inoculum on the plate surface in three different planes. The inoculated plates were then placed at room temperature to allow absorption of excessive moisture. With sterile forceps selected antibiotic disks (Vancomycin (30 µg), Bacitracin (30 µg) and Optochin (50 µg)) were placed on the inoculated plates and incubated at 37°C for 24 hrs in an inverted position. After this period of incubation, the diameters of inhibition zones were noted and measured by a ruler in (mm), results were

determined according to the National Committee for Laboratory Standards [16].

Identification by Latex Test (PASTOREX® STREP)

This test is a rapid, sensitive agglutination test for grouping of (α or β) haemolytic streptococci (which were grown on the surface of blood agar plates) belonging to the main lancefield groups. The test involves the use of latex suspensions specific for group A, B, C, D, F and G.

Identification of β or α -haemolytic streptococci based on group-specific polysaccharides requires previous extraction of these antigens from colonies obtained by primary blood agar cultures. With the PASTOREX® STREP system, this requires only 15 minutes at room temperature or 10 minutes at 37°C. Extraction is achieved by an active enzyme that causes lysis of the cell wall.

In the presence of the antigen, the latex particles coated with homologous antibodies agglutinate very rapidly. The speed of agglutination depends on the sensitivity of the latex particles suspensions, which are governed by the quality of the antisera raised in rabbits using lancefield's immunization protocol and by the amount of purified immunoglobulins adsorbed on the latex particles.

Results and Discussion

Fifty plaque samples were collected from teeth of the staff of Biotechnology-Department in the College of Science, Al-Nahrain University. Isolation of *Streptococcus* species was done using selective enrichment technique including culturing of cells on MS-agar (Mitis-Salivarius agar), which promotes growth of streptococci and suppress other bacterial species [7]. Accordingly forty five samples were considered to be positive bacteria about (10^4 cells/ml) [10].

Single colonies from the surface of MS-agar were selected for further purification by subculturing on the surfaces of blood agar and MS-agar media.

Isolates were first identified depending on their gram-staining, microscopic examination and catalase test. The streptococci are gram-

positive, individual cocci which are spherical or ovoid and are arranged in chains under light microscope and may be considered as catalase negative bacteria as indicated by identification scheme of Friedrich [10].

Accordingly, forty three isolates were found to be true streptococci (showed positive results) and three isolates showed negative results.

Isolates were also identified depending on the colonial shape and form on the surface of MS-agar media. Isolates could be varied between, hard coherent, raspberry like high refractile, raised colonies that were identified as *S. mutans* which was considered as one of the most important etiological agent of dental caries [17], while other colonies showed characteristics of zooglycic form, which were firmly attached to agar which were considered as *S. sanguis* depending on Colman and Williams, [18] identification scheme. Other type of colonies produced a minute circular forms were considered as *S. salivarius*, the colonies that were little raised with mucoid texture were considered as *S. milleri* following the information of Buchanan and Gibbons [19]. Accordingly, thirty nine isolates were suspected to belong to mutans streptococci group and four isolates were belonged to *S. sanguis*, *S. salivarius* and *S. milleri*.

Another characterization for the previous forty three isolates depended on their capability of producing specific type of exopolysaccharides as a criterion for identification scheme separating species of mutans streptococci group bacteria and *S. sanguis*, from that *S. salivarius* which produce polyfructan (levans). Results shown that only thirty seven isolates were capable of producing polyglucan, while the other six isolates were considered as levans producers, since no reaction was obtained, when they were mixed with ethanol and methanol and this was a good sign for the polyfructan-production as determined from the description of Guthof [11].

A rapid differentiation between colonies belonged to mutans streptococci group from that of other streptococci (*S. sanguis*) on MS-agar media. If the 2,3,5-triphenylterazolium chloride (TTC) was reduced, it would stain the mutans streptococci

colonies to dark pink, due to mannitol 1-1-phosphate dehydrogenase-mediated hydrolysis of mannitol [20] to the acid by these bacteria [12]. Results indicated that thirty five isolates were stained with dark pink color and considered to be (positive result) and two isolates showed (negative result) no change in color.

For further identifications of the oral *Streptococcus* species, other biochemical test were included. One of those is the bacterial isolates to grow in a culture media containing (4%) NaCl. Results showed different patterns of growth, varied between a good growth with a percentage of (63%) to a weak growth with a percentage of (27%) and no growth with a percentage of (10%).

The tolerance of 4% NaCl was considered as a criterion to differentiate species of the oral *Streptococci* from that associated with dental disease [21]. Only thirty three isolates with a percentage of (90%) (which gave the good and weak growth in 4% NaCl) were taken for further identification.

The ability of the isolates for utilizing various carbohydrates was carried out in growth media containing (mannitol, sorbitol and inulin) with sucrose as (a positive control). Results showed that most isolates were capable of reducing mannitol sugar with the exception of three isolates as compared with the sucrose (positive control) by changing the color of the media from red to yellow. Seven isolates were incapable of fermenting sorbitol and inuline sugars.

Beighton *et al.*[22] demonstrated that all the mutans streptococci group that were isolated from humans are capable to ferment mannitol, sorbitol and inulin sugars except for *S. sobrinus* and *S. cricetus* in which 11-89% of strains are positive to sorbitol and inulin. So that three isolates did not belong to mutans streptococci group because of their inability to ferment mannitol, sorbitol and inulin and five isolates may belong to *S. sobrinus* and *S. cricetus* bacteria because of their ability to ferment mannitol with inability of fermenting sorbitol and inulin

Antibiotic sensitivity test may be used as a criterion for separating oral *Streptococcus* from that belongs to *Streptococcus pneumoniae* and *Pediococcus spp.* Results

shown in Table (1) indicates that most isolates were resistant to bacitracine and optochin and vancomycin with the exception of seven isolates (N₁, N₃, N₆, N₇, N₈, N₂₆ and N₂₈), three isolates (N₆, N₈ and N₂₈) and seven isolates (N₆, N₈, N₂₂, N₂₅, N₂₈, N₃₀ and N₃₄) isolates were sensitive to these antibiotics respectively. The resistance pattern to vancomycin antimicrobial agent for the four isolates (N₂₂, N₂₅, N₃₀ and N₃₄) is due to the reasons that described previously by Marsh, [23].

In order to confirm the biochemical tests and their results that had been done and recorded for bacterial isolates., identification was made finally by Latex test (Pastorex^RStrep) according to the method that was described by the manufacturing company (Bio-Rad France).

After the appearance of the agglutination reaction result shows in Fig. (1) indicated that all isolates were related to the genus *Streptococcus* with the exception of three isolates (N₆, N₈ and N₂₈), since no agglutination reaction was obtained for these isolates. These three isolates were represented a percentage of about (9.09%) from the whole thirty three isolates.

Grouping of the identifiable isolates was done as follow:

1. Six isolates were related to (serotype A) which
2. Represented (18.18%) from the whole isolates.
3. One isolate was related to (serotype B) and represented (3.03%). Eight isolates were related to (serotype C) and represented (24.24%).
4. Eight isolates were belonged to (serotypes D) and represented (24.24%).
5. Two isolates were related to (serotype F) and represented (6.06%).
6. Five isolates were related to (serotype G) and represented (15.15%).

The identifiable bacteria by this test are not only related to the genus streptococci but also to the mutans streptococci bacteria because they were been isolated from specimens of human dental plaque. Killian *et al.*[24] found that mutans streptococci bacteria was responsible for the initiation of the dental

plaque formation on the tooth surface even after four hours of cleaning the teeth.

Beighton *et al.*, [22] separated mutans streptococci group bacteria into eight serotypes according to their surface antigens in their cell walls. The classification series ranged from serotypes A to G and / or H. these serotypes can in turn be classified into four groups according to another biochemical properties [25] and to the specificities of their enzymes in extracellular polysaccharide synthesis [16].

Thus serotypes D, G and / or H (*S. sobrinus*) form one group, Serotype A (*S. cricetus*) another, serotype C, E and F (*S. mutans*) the third and serotype B (*S. rattus*) the fourth group. According to this information, isolates can in turn be classified into the following:

1. Six isolates were related to the mutans streptococci (*S. cricetus*) (serotype A) represented (18.18%).
2. One isolates was related to mutans streptococci (*S. rattus*) (serotype B) represented (3.03%).
3. Ten isolates were related to (serotype C) and (serotype F) were related to mutans streptococci (*S. mutans*) with a percentage of (30.302%).
4. Thirteen Isolates were related to (serotype D) and (serotype G) were belonged to mutans streptococci (*S. sobrinus*) with a percentage of (39.294%).

From these results, the most isolated species of mutans streptococci bacteria from the previously isolated dental plaque human specimens were (*S. sobrinus*) followed by (*S. mutans*) according to the percentage ratio of each of them.

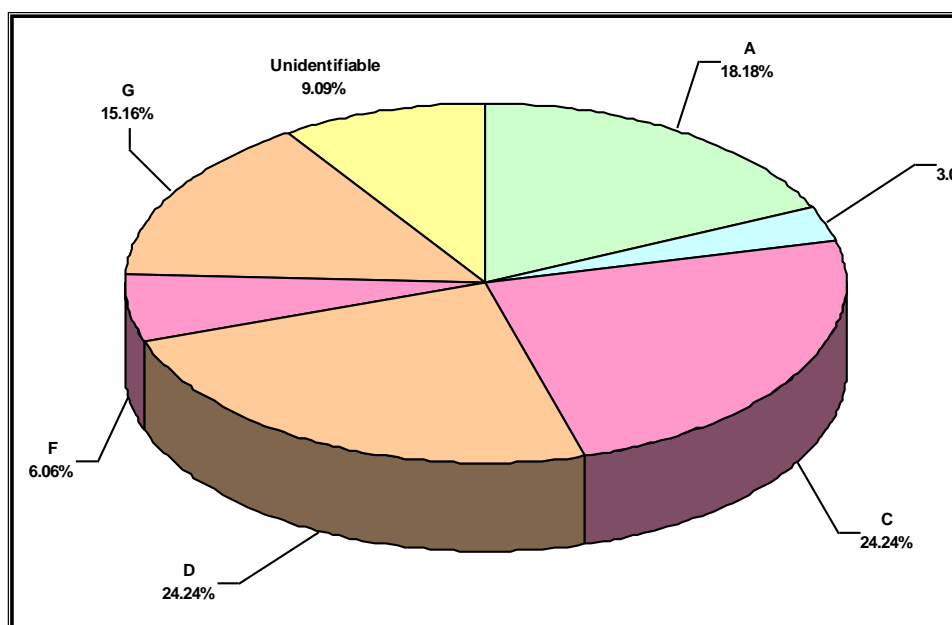


Fig. (1) : Percentages of each Lancefield group among the Streptococcal isolates.

Table (1)
Antibiotic sensitivity test of Streptococcal isolates.

No.	Symbols	Bacitracin	Vancomycin	Optochin
1	N ₁	S	S	R
2	N ₂	R	S	R
3	N ₃	S	S	R
4	N ₄	R	S	R
5	N ₅	R	S	R
6	N ₆	S	R	S
7	N ₇	S	S	R
8	N ₈	S	R	S
9	N ₉	R	S	R
10	N ₁₀	R	S	R
11	N ₁₁	R	S	R
12	N ₁₂	R	S	R
13	N ₁₃	R	S	R
14	N ₁₄	R	S	R
15	N ₁₅	R	S	R
16	N ₁₆	R	S	R
17	N ₁₇	R	S	R
18	N ₁₈	R	S	R
19	N ₁₉	R	S	R
20	N ₂₀	R	S	R
21	N ₂₁	R	S	R
22	N ₂₂	R	R	R
23	N ₂₃	R	S	R
24	N ₂₄	R	S	R
25	N ₂₅	R	R	R
26	N ₂₆	S	S	R
27	N ₂₇	R	S	R
28	N ₂₈	S	R	S
29	N ₂₉	R	S	R
30	N ₃₀	R	R	R
31	N ₃₂	R	S	R
32	N ₃₃	R	S	R
33	N ₃₄	R	R	R

R: Resistance

S: Sensitive

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الخلاصة

عزلت خمسين عينة من الطبقة المغشية لسطح السن. خمس وأربعون عينة أظهرت نمواً إيجابياً للبكتريا حوالي (10^4 بكتريا/مل) على سطح الوسط المخصص للنمو (Milis-Salivarius agar medium). ثلاثون عينة إعتبرت عائدة لبكتريا *Streptococcus* وخاصة للمكورات الفموية والتي تباينت بين الأنواع *S. sobrinus* و *S. mutans* (serotype C,F) و *S. cricetus* (serotype A) و *S. rattus* (serotype B) وينسب () 39.39% و 30.30% و 18.18% و 3.03% تباعاً والتي تم تشخيصها اعتماداً على الطرق البايوكيميائية وخصوصية عالق ال (Latex) المضاد والخاص بالمجاميع (A و B و C و D و F و G) المتعددة السكريات (المستضاد) والموجودة على الجدار الخارجي لهذه البكتريا (group) Lancefield أو (Serotype group).