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Plasmid Profile of Locally Isolated *Streptococcus mutans* in Relation with Mutacin Production

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Abstract: The present study was aimed to isolate and identify *Streptococcus mutans* from dental caries, and ability of bacterial isolates for mutacin production and selecting the efficient producer isolate and studying plasmid profile for the selected isolate and its role in mutacin production. In order to isolate *Streptococcus mutans*, 80 swab samples were collected from dental caries of patients attends Al-Dora health center and Al-Zewiya health center in Baghdad city. From these samples 80 bacterial isolates were obtained. Results of identification depending on morphological, cultural and biochemical tests showed that ten of these isolates were belonged to *Streptococcus mutans*, then results of identification were confirmed by using VITEK-II identification system. All isolates were screened to examine their ability in mutacin production. Plasmid profile of *S. mutans* S2 was studied to investigate its role in mutacin production. Results showed that *S. mutans* S2 has only one plasmid DNA band. This plasmid was not responsible for mutacin production according to the results of curing experiment by using interchalating ethidium bromide dye in a concentration of 800 µg/ml. Cured cells were still have the ability of mutacin production which indicated that this trait is chromosomally encoded.

Key words: Plasmid profile, *Streptococcus mutans*, mutacin

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Introduction

Genus *Streptococcus* comprises important pathogens that have a severe impact on human health (1), the genus *Streptococcus* includes the pyogenes ic, oral and anaerobic groups of streptococci, as well as a group of other streptococci. The streptococci are catalase-negative, and they commonly attack red blood cells, with either greenish discoloration (α-hemolysis) or complete clearing (β-hemolysis) (2). *Streptococcus mutans* is a Gram-positive bacterium, which plays a key role in the formation of the dental plaque biofilm as an early coloniser (3). The primary habitats for *S. mutans*aremouth, pharynx, and intestine (4). Virulence factors in *S. mutans*are mutacin that increase in bacterial resistance to antibiotics impels the development of new anti-bacterial substances. Mutacins (bacteriocins) are small antibacterial peptides produced by *Streptococcus mutans* showing activity against bacterial pathogens. (5). Types of mutacin are Mutacin I, bacteriocins, Mutacin H-29B,Mutacin III, Mutacin IV, Mutacin B-Ny266, Mutacin 1140, and Mutacin N (6). Plasmids are extrachromosomal genetic elements which are widespread in bacteria, they

have their origin of replication, and autonomously replicates with respect to chromosomal DNA and stable inherited (7). Plasmids influence the biology of the host, sometimes dramatically. Plasmids also display great diversity in size, mode of replication and transfer, host range, and the set of genes they carry, making them interesting elements for analysis. Many studies already known that acridine orange, ethidium bromide and SDS affect plasmid replication. Some physical effects also influence plasmid replication like elevated growth temperature and thymine starvation (8, 9). The efficiency of curing generally varies from less than 0.1% to more than 99% depending upon the element involved, the bacterial strain, and the mode of action of the curing agent(9). It is generally assumed that curing activity is related to the ability of these compounds to intercalate into superspiralized DNA and to inhibit its replication (10). Because of the limited studies and importance of both (*Streptococcus mutans* and mutacin as " designer drugs" alternatives to traditional antibiotics that target specific bacterial pathogens) in genetic engineering, agricultural, biological control, and medicine. The present work was aimed to isolate and identify *Streptococcus mutans* from dental caries, screening the ability of bacterial isolates for mutacin production and selecting the efficient producer isolate and studying plasmid profile for the selected isolate and its role in mutacin production.

Materials and methods

Collection of sample

Eighty samples were collected from patient with different dental caries (pit, fissure and dental roots) during the period from November 2013 to January 2014. Those patients were attends Al-dora health center and Al-Zewiya health center in Baghdad city.

Isolation of *Streptococcus mutans*

Bacterial isolates obtained from dental caries samples were streakedon selective medium (Mitis Salivaris agar) for isolation of *S. mutans*, then plates were incubated at 37°C for 48 hours under anaerobic conditions.

Identification of *Streptococcus mutans*

Bacterial isolates were identified according to their morphological and cultural characteristics and biochemical tests. Morphological and cultural characteristics includes colony size, shape, color, and odor of the bacterial isolates were studied on Mitis Salivaris agar prepared, plates were incubated at 37°C for 24 hours under anaerobic conditions. Biochemical tests included catalase test, blood hemolysis test and carbohydrate fermentation test. All these tests were done according to Collee *et al.,* (1996).

Identification by using VITEK-II identification system

The innovative VITEK-II microbial identification system includes an expanded identification database, the most automated platform available, rapid results, improved confidence, with minimal training time. Bacterial isolates that suspected to be *S. mutans* were completely identified by using VITEK-II identification system.

Screening bacterial isolates for Mutacin production

Bacterial isolates identified as *S. mutans* were examined to detect their ability in mutacin production.

Induction of Mutacin production

Induction of mutacin production by the bacterial isolates was achieved by supplementing Brain Heart Infusion broth as production medium with yeast extract 2% (w/v) and $CaCO_3$ 1% (w/v), then cultures were incubated at 37°C for 24 hours under anaerobic conditions. After incubation cultures incubated at 70 °C for 10 minutes to kill the cells and inhibit protease activity, then centrifuged at 6000 rpm for 10 minutes (12). Supernatant was regarded as a crude mutacin.

Plasmid profile

Plasmid profiles for the selected isolate of *S. mutans* was studied after extraction by using *AccuPrep*® Plasmid Mini Extraction Kit supplied by Bioneer / Korea.

Agarose Gel Electrophoresis (13)

Plasmid DNA samples extracted from the selected isolate of *S. mutans* were loaded into the well of agarose gel (0.7%) after mixing with loading buffer prepared, then gel electrophoresis was run horizontally in Tris-borate EDTA (1X TBE buffer) prepared for 2-3 hours at 5v/cm. After electrophoresis, gel was stained with ethidium bromide in concentration of 0.5 µg/ml for 30-45 min, then DNA bands were visualized under UV transilluminator.

Curing of plasmid DNA

Curing experiment was achived according to Zaman *et al.,* (14). Investigate the role of plasmid of *S. mutans* plasmid in mutacin production. Ethidium bromide was used as curing agent. Bacterial cells of the selected isolate was grown in Brain Heart Infusion broth to mid log phase, then 0.1 ml inoculums of the growth culture were used to inoculate series of tubes contaninig 5 ml Brain Heart Infusion broth with different concentrations of ethidium bromide (0, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 µg/ml), then all tubes were incubated at 37°C for 24 hours. Growth density for different tubes was observed by nacked eye and compared with control to determine the effect of ethidium bromide on growth of bacterial cells. The sub-lethal concentration of agent that inhibits the growth of bacterial cells considered as the minimum inhibitory concentration (MIC). Samples were taken from tubes containing the highest concentration of

ethidium bromide that still allow bacterial growth, and diluted appropriately, then 0.1 ml from the proper dilution was taken and spread on Brain Heart Infusion agar plates and incubated overnight at 37°C. One hundred of surviving colonies were analyzed for the presence or absence of antibiotic resistance traits as a result of plasmid curing. Those colonies were replicaplated (using toothpick) on Brain Heart Infusion agar (master plate), and on Brain Heart Infusion agar plates containing antibiotics to which the original isolate was resisting. If the colonies were able to grow on the master plate, but not on the selective agar containing the appropriate antibiotic, it means that cells of this colony are cured cells that lost the resistance of this antibiotic. These cured colonies were tested for their ability to inhibit of *S. pyogenes* growth, in addition to investigate the presence of plasmids in each colony.

Results and Discussion

Collection of samples

In order to isolate *Streptococcus mutans*, eighty samples were collected by taking swabs from the mouth cavity of patients suffering from dental caries. These samples were put into peptone water and then streaked on semi-selective Mitis Salivaris agar medium (MSA). Bacterial isolates were then streaked on Brain Heart Infusion agar plates and incubated at 37°C under anaerobic conditions. Colonies of these isolates appeared as points getting into the culture medium and surrounded by a white opaque halo. Bacterial isolates grown on MSA were then streaked another time on selective Mitis Salivaris Bacitracin agar medium (MSBA), MSBA medium was composed of MSA and 20% sucrose and 0.2 U/ml of Bacitracin which inhibited the growth of most bacteria excepted *S. mutans* and *S. sorbinus*. The inclusion of sucrose in this medium leads to the formation of glucan and distinctive colony appearance that aids identification of *S. mutans* (2, 15).

Identification of bacterial isolates

Bacterial isolates grown on selective medium (MSBA medium) and suspected to *S. mutans* were identified according to their morphological and cultural characteristics and biochemical test.

Morphological and cultural characteristics

Bacterial isolates grown in Mitis Salivaris Bacitracin agar medium were first identified according to their morphological and cultural characteristics .Results showed that these isolates are gram positive, spherical or ovoid cells. Colonies of these isolates are highly convex, raised, light – blue, frosted glass appearance with either rough or smooth surface. These colonies are also highly adherent to the agar surface if it's picked up by the loop, polysaccharide parameters were observed as a glistening drop on top of the colony or as a pool besides the colony.

Biochemical identification

Bacterial isolates suspected to be *Streptococcus* spp were subjected to examine their biochemical characteristics. Results indicated in Table 1 showed that these isolates are gram positive, negative for catalase and they were able to ferment mannitol, sorbitol and raffinose sugars. These isolates were unable to produce hemolysin exhibiting gamma hemolysis on blood agar medium. According to these results, these isolates were regarded as *S. mutans*.

Table (1): Results of biochemical tests for identification of *S. mutans*.

Biochemical test	Isolate No.									
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
Gram staining	+	+	+	+	+	+	+	+	+	+
Catalase test	-	-	-	-	-	-	-	-	-	-
Blood hemolysis	γ	γ	γ	γ	Γ	γ	γ	γ	Γ	γ
Acid production from										
mannitol	+	+	+	+	+	+	+	+	+	+
Sorbitol	+	+	+	+	+	+	+	+	+	+
Raffinose	+	+	+	+	+	+	+	+	+	+
Salt tolerance										
4% NaCl	+	+	+	+	+	+	+	+	+	+
6.5% NaCl	-	-	-	-	-	-	-	-	-	-

To confirm identification of bacterial isolates as *S. mutans*, biochemical characteristics of these isolates were examined also by using VITEK-Π. Results showed that *S. mutans*were negative to hydrolysis of Urease (URE) and Arginine dihydrolase1 (ADH1) and positive to Alpha-Glucosidase (AGAL) and fermentation of Lactose (LAC).

Screening of *S. mutans* isolates in mutacin production

Locally isolated *S.mutans* were screened in order to select the efficient isolate in mutacin production. The ability of these isolates in mutacin production was assayed after culturing at 37°C in Brain Heart Infusion broth medium and incubation till the optical density was reached 0.8, then crude mutacin in culture filtrate was used to study the antagonistic effect against the test microorganism (*Streptococcus pyogenes* and *Escherichia coli*) by measuring the inhibition zones according to well diffusion method. Results indicated in Table 2 showed that all of the 10 isolates of *S.mutans* were mutacin producers according to the inhibition zones against *S. pyogenes* as shown in Figure 1. Diameter of inhibition zones were ranged between 7 and 20 mm.

Among these isolates *S.mutans* S2 was the best in mutacin production which gave the maximum diameter zone of inhibition (20 mm) against *S. pyogenes*. According to these results, this isolate was selected to study the optimum condition for mutacin production. On the other hand, it was found there is no any inhibitory effect of mutacin produced by local isolates of *S. mutans* on the second test microorganism (*E. coli*). This may be due to the nature of *E. coli* cell wall that haven't specific receptor for mutacin in addition to the selectivity of cell membrane toward different harmful molecules in culture medium as mentioned by Mota-Meira *et al.*, (16) which showed that most gram negative bacteria were resistance to mutacin. Also, Mota-Meira *et al*. (17) showed that mutacin was active against Gram-negative bacteria only after treatments altering the outer membrane thus giving access to the cytoplasmic membrane.

Table (2): Ability of local isolats of *S. mutans* in mutacin production termed by inhibition zones against *S. pyogenes*.

Isolate symbol	Inhibition zone (mm)
S1	18
S2	20
S3	15
S4	10
S5	7
S6	14
S7	10
S8	15
S9	17
S10	9

Figure (1): Inhibitory effect of mutacin produced by locally isolated *S. mutans*S2 against *S. pyogenes* on brain heart infusion agar medium after incubation at 37°C for 16 hours under anaerobic conditions.

Plasmid profile of *S. mutans*

Plasmid profile of *S.mutans* S2 was studied to determine its role in mutacin production, by using *AccuPrep*® Plasmid Mini Extraction Kit for extraction of plasmid DNA. Since most mutacin of G+ are plasmid encoded (18). Result shown in (Figure 2) indicates that the locally isolated *S. mutans* S2 has a plasmid DNA after electrophoresis on agarose gel. This result was agreed with Caufield *el al.,* (18) who mentioned that 5.6-kb plasmid in *S. mutans* was thought to be related to mutacin production, because most bacteriocins of gram-positive bacteria are plasmid encoded. Because of its high sequence variability in the hypervariable region (HVR) and its low prevalence, the cryptic plasmid is a useful epidemiological marker for studying transmission. A cryptic plasmid resides in ~5% of the isolates of *S. mutans*. The function of this plasmid remains unknown, although its sequence has been published (19).

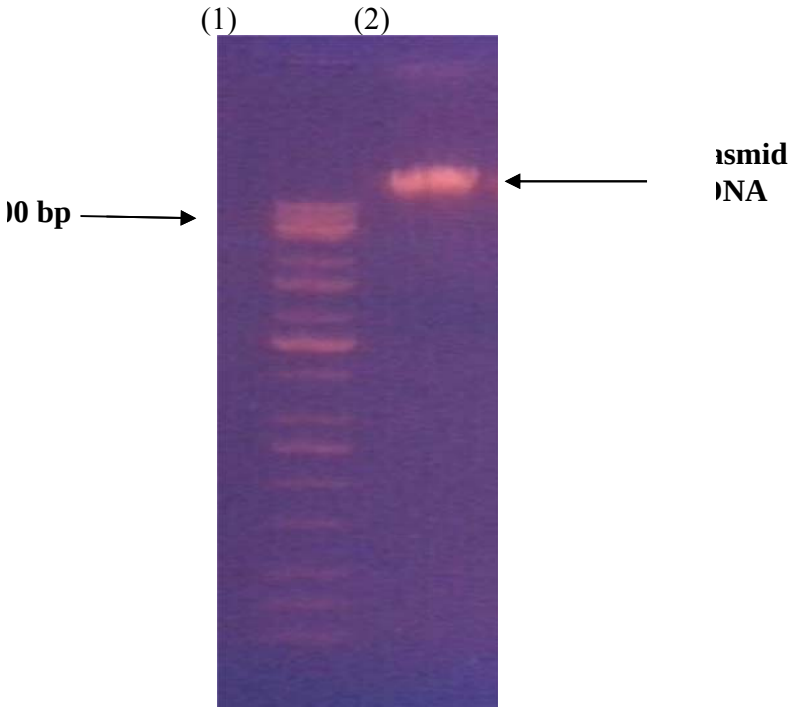

Figure (2): Plasmid profile of the locally isolated *S. mutans* S2 on agarose gel (0.7%) after electrophoresis at 5v/cm for 3 hrs.
Lane (1): DNA ladder marker (2500bp), Lane (2): *S. mutans*

Curing of plasmid DNA

An attempt to cure plasmid DNA of the locally isolated *S. mutans* S2 was achived by using ethidium bromide as a curing agent to evaluate the role of this plasmid in mutacin production.

Table (3): Effect of ethidium bromide on growth of *S. mutans* S2 after incubation at 37°C for 24 hours

Concentration (µg/ml)	Bacterial growth
0	+++
50	+++
100	+++
200	+++
300	++
400	++
500	++
600	++
700	+
800	±
900	_
1000	_

(+++):Very good growth; (++): Good growth; (+): moderate growth
(±):Slight growth; (-): No growth

Results indicated in Table 3 showed that the locally isolated *S. mutans* S2 was still able to grow in brain heart infusion broth in presence of gradual concentration of ethidium bromide till the concentration of 800µg/ml, (a concentration which was regarded as a sub-lethal concentration). From culture medium containing this concentration of ethidium bromide (800 µg/ml), 100 µl was taken, diluted, and spread on Brain Heart Infusion agar plates, and incubated at 37°C for 24 hours. Then 100 colonies were selected randomly to examine their antibiotic resistance on selective medium contining antibiotic to which wild type is resist to determine the cured colonies (which cannot able to grow on these antibiotic contining media). Results showed that all colonies are still able to grow on medium containing erythromycin and bacitracin. One of these colonies was selected and examined for the presence of its own plasmid by extraction of the plasmid DNA and electrophoresis on agarose gel. Result shown in Figure 3 indicates that this colony of *S. mutans* S2 was lost their own plasmid. Cured cells of this colony were examined for the ability to produce mutacin by growing under the optimum conditions for 24 hours at 37°C under anaerobic condition. Results showed that these cured cells were still able to produce mutacin in the culture medium because of the inhibitory effect of mutacin produced in crude filtrate against the test microorganism (*S. pyogenes*). These results declared that *S. mutans* S2 plasmid was not responsible for mutacin production; furthermore, this trait is chromosomally

located. This result was agreed with Kamiya *et al*. (20) who mentioned that the location of the genes that responsible for mutacin production is chromosomally located in *S. mutans*. (21) obtained same result when studied on *S. mutans.*

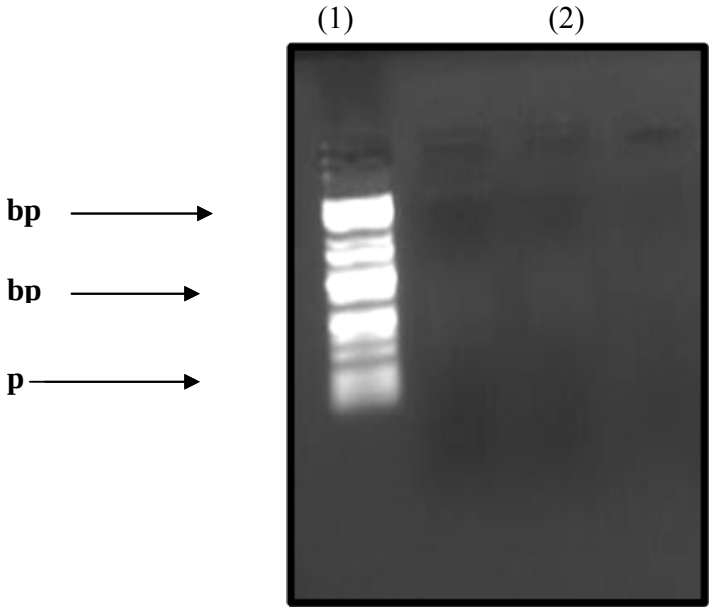

Figure (3): Plasmid profile of cured cells of *S. mutans*S2 on agarose gel (0.7%) after electrophoresis at 5V/cm for 3 hrs
Lane (1): DNA ladder marker (2500bp), Lane (2): *S. mutans*

Conclusions

The present study concluded that plasmid was not responsible for antibiotic resistance and for mutacin production according to the results of curing experiment by using interchalating ethidium bromide dye in a concentration of 800 µg/ml. Cured cells were still have the ability of mutacin production which indicated that this trait is chromosomally encoded.

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