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Isolation and identification of *Streptococcus mutans* from dental caries patients at Thi-Qar province/Iraq

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Abstract:

Dental caries is one of an important common human infectious disease that can lead to loss of tooth structure, it is occur due to the metabolic activation of the plague microorganisms especially *Streptococcus mutans* bacterium. Eighteen samples from saliva and dental plaque were collected from patients with dental caries active at ages from (7-25) years from Specialized dental hospital and dental clinics at Thi-Qar province during the period from November to December 2016.Thirty-three isolates belong *Streptococcus mutans* were identified by cultural methods, biochemical tests and Api 20 Strep. system, while twenty-eight isolates identified by using Polymerase Chain Reaction (PCR) technique and sequencing of 16S rRNA gene with 507 bp, whereas by using 16S rRNA gene the result confirmed that these isolates were belong to *Streptococcus mutans.* The aim of study is Isolation and identification of *Streptococcus mutans* bacterium from the dental caries and dental biofilm patients at Thi-Qar province by of 16S rRNA gene.

Keywords: *Streptococcus mutans*, 16S rRNA gene, PCR.

1. Introduction:

Oral cavity of human is considered proper sterile at birth (Ion, 2013), but after 2-5 days a microbes begin to enter the mouth through feeding until it is reach during few months to more than a million bacterial cell. Most of them are harmless, but a under certain condition, about 12-17 different bacterial species have been associated with dental plaque (Metwalli, *et al*., 2013).

Dental caries is one of an important common human infectious disease that can lead to loss of tooth structure, thereby changes in functions of mouth (Schwendicke *et al*., 2015). It is occur due to the metabolic activation of the plague microorganisms, especially *Streptococcus mutans* bacterium, whereas these organisms are able to ferment the dietary carbohydrates(e.g. sucrose and glucose), which are remains between the teeth, for production the organic acids that lead to breakdown the hard tissue(enamel, dentin and cement) due to remove minerals of tooth surface demineralization process (Veale *et al*., 2016). Several previous clinical studies have shown that *S. mutans* is considered as a primary etiological factor for dental caries (Saravia *et al*., 2011; Marsh *et al*., 2015). Also detection and identification the *S.mutans* among other pathogens of dental caries may be important for not only predicting and prevention of tooth decay, but also this bacterium is associated with infective endocarditis mouth , skin, muscles, and central nerve system ((Nomura *et al*., 2006; Nobbs *et al*.,2009). In the present study, identification of *S.mutans* not only relying on traditional methods which are usually determine from phenotypic characteristics such as colony morphology, carbohydrates fermentation, and API 20 Sterp testing, but we also used the PCR technique to confirm the presence of the 16S rRNA genes in all isolates and study the nucleotides sequencing of 16S rRNA gene.

Polymerase chain reactions (PCR) technique has high specificity and sensitivity (Petti *et al*., 2005), therefore it is currently being applied in a wide range of medical diagnostics and research (Cai, *et al*., 2016). The occurrence of several genes copies of 16S rRNA in the cell and the key role of this genetic target for the PCR detection of bacteria in all different field of microbiology (Al-Ahmad, *et al*., 2006).

2. Materials and Methods:

Samples collection:

Saliva and dental plaque samples from eighteen dental patients suffering from dental caries at ages from (7-25) years from both sexes in Specialized Dental Hospital and Dental Clinics at Thi-Qar province during

the period from November to December 2016 were collected. Each individual was asked to chew a piece of Arabic chewing gum (0.4-0.5g) for five minutes to stimulate salivary collection as much as possible, then saliva a proximally (2-3ml) was collected in sterilized tube. While the dental plaque samples were collected by using sterile curettes (Gracey Curettes). Supragnigival plaque was taken first from the crown site, and then was taken from the sub-gingival plaque, the same procedure was applied to the plaque samples obtained from the natural tooth, and the plaque samples were suspended in 1 ml of sterile Phosphate buffer saline PBS, the two type of samples were transported on icebox to the laboratory. Saliva and plaque samples were collected under standard conditions according to Hadi, 2011.

Isolation of *S.mutans*:

The samples were homogenized by vortex mixer for two minutes. Tenfold serial dilutions were prepared by using normal saline. Two dilutions were selected for each microbial type and inoculated on the Mitis Salivarius Bacitracin Agar (MSB agar), the selective media for mutans streptococci, whereas 0.1ml was withdrawn from dilutions (10^3-10^5) and then spread in duplicate by using sterile microbiological spreader on the plates of MSB agar then the plates were incubated anaerobically by using an anaerobic jar for 48 hrs. at 37 ^oC followed by aerobic incubation for 24 hrs. at 37 ^oC (Hadi, 2011).

Traditional Methods for Identification of *S.mutans*: (Hadi, 2011).

After estimation of positive samples on the surface of MSB medium, small colonies were subcultured on the surface of blood-agar plates for further purification and incubated anaerobically for two days at 37 ^oC. The following methods were used for initial characterization of the isolates:

- Morphology of colony on MSB agar and blood agar.
- Gram-staining and microscopic examination.
- Catalase test.
- Carbohydrates fermentation
- Rapid API 20-Strep.

Polymerase Chain Reaction:

Polymerase chain reaction (PCR) was used to confirm the presence or absence of the 16SrRNA genes in the 33 isolates, one colony of each bacterium from an agar plate was used as the template, genomic DNA was extracted from *Streptococcus mutans* bacterial isolates by using Genomic DNA Mini Bacteria Kit, all primers used in detection 16SrRNA genes were designed by using NCBI Gene-Bank and Primer one online and providedby,,,,,(BioneerCompany,Korea).Forward,,,,,primer,,,,CCACACTGGG-ACTGAGACAC and Reverse primer GTTTACGGCGTGGACTACCA. PCR was performed with 5µL of template DNA in a total reaction volume of 20 µl consisting of 1.5µL of Forward Primer, 1.5µL Reverse Primer (12µL) Nuclease free water, the PCR program consisted of 30 cycles of denaturation (95 ^oC for 30 sec.), annealing (58 ^oC for 30 sec.), and extension (72 ^oC for 1 min) and a final extension step at 72°C for 5 min to amplified 16SrRNA, the positive result of 16S rRNA gene was confirmed by 1% agarose gel electrophoresis, then electric current was performed at 100 volt and 80 AM for 1hour.PCR products were visualized by using UV Trans illuminator (Sato *et al*., 2003).

3. Results:

In the present study, a total 80 samples of saliva and plaque from patients suffering from dental caries and dental plaque have been collected and tested during period from November to December 2016.only 33 samples are given growth for *S.mutans* this is about 41.2% of patients as in figure (1).

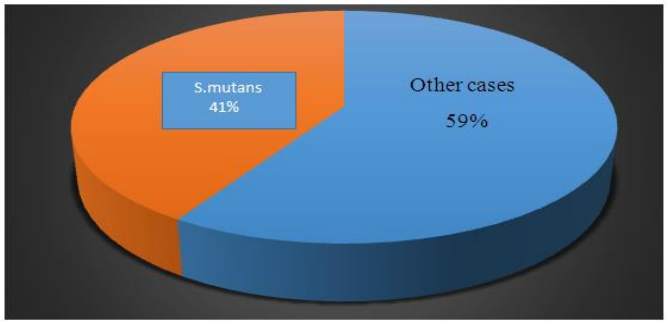

Figure 1: The occurrence of *S.mutans* isolated from 80 saliva and dental plaque samples

Identification of *S.mutans* was carried out by 5 stages:

3.1 Morphology of Colony:

One the selective MSB agar plates, *S.mutans* colonies appeared pale-blue in color about 2-3mm in diameter as avoid or spherical in morphology with raised or convex surface adhered well to the medium surface. Some colonies of appeared as irregular colonies with rough or frosted-glass surface appearance (rough colonies), while others appeared with smooth

surfaces colonies (smooth colonies). Most of *S.mutans* colonies had a depression at the middle of the colony containing a drop of polysaccharide, or sometimes the whole colony submerged in a pool of polysaccharide as in figure (2).

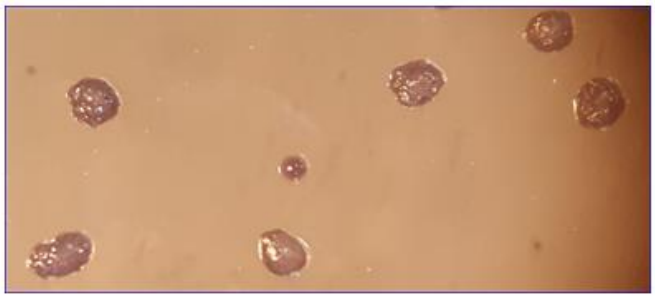

Figure 2: *S.mutans* colonies growth on MSB agar

3.2 Microscopic Examination:

This results of this test have been showed that all *S.mutans* (33 positive sample were gram positive, avoid or spherical in their shape, arranged in short or medium length chain as in figure (3).

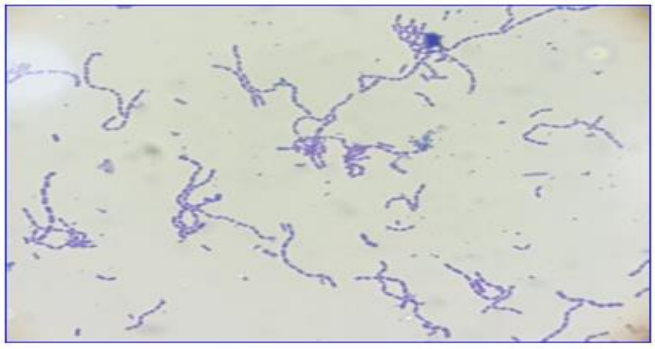

Figure 3: Gram stain of *S.mutans* cells

3.3 Biochemical tests:

The results of biochemical tests showed that all isolates were negative for catalase test and had the ability to formentation of innulin, while it is gave positive results for hydrolysis of bile esculin and fermentation of manntol, sorbitol, sacrose, and Raffinose as shown in table (1).

Table 1: Biochemical tests of *S.mutans*

NO.	Biochemical test		Result
1	Catalase test		+
2	Hydrolysis of bile isculin		+
3	A bility to ferment carbohydrates	Mannitol	+
		Sorbitol	+
		Raffinose	+
		Sucrose	+
		Innulin	-

3.4 A PI 20-Strep.System:

The result of Api-20 Strep. test has revealed that only 33 isolates from 80 samples were identified as *S.mutans* as in figure(4).

Figure 4: Calculate the numerical profile in Api Sterp. system (+) The test positive (-) the test negative (5240550) and (5040730) are the numerical profile.

3.5 Detection of *S.mutans* by 16S rRNA gene:

A total of 33 isolates of *S.mutans* which identified by conventional methods such as; morphology of coloine, microscopic examination, biochemical tests and API 20 strep. were subjected to DNA extraction and PCR assay for presence of 16S rRNA gene. The results demonstarted that 28 (84.8%) of isolates had 16S rRNA gene with band 507 pb. as shwon figure (5).

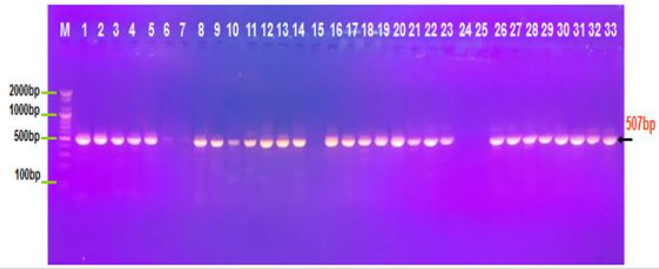

Figure 5: Agarose gel electrophoresis image that shown the PCR product of 16S rRNA gene in *Streptococcus mutans* isolates. Where M: Marker (2000-100bp), lane (6, 7, 15, 24 and 25) only negative amplification for 18S rRNA gene, at (507bp) PCR product

Dental caries remains the most prevalent disease worldwide, burdening billions of people, especially children, with pain and subsequently poorer quality of life and general health (Schwendicke *et al*., 2015).

S.mutans a primary etiologic agent of human dental caries, is especial pathogen at forming biofilms

on the hard tissues of the human oral cavity. The ability of *S.mutans* to biofilm formation on teeth surface, dental caries to synthesize extracellular glucans is a critical virulence factor involved in the pathogenesis of dental caries in humans and animals.

The present study deals with *S.mutans* isolation and identification, these were done according to cultural features, microscopic examination, biochemical tests and using the Api 20 Strep system which is specific for identification of Streptococcaceae from other bacteria. In addition, the final identification of *S.mutans* was based on present of the 16S rRNA gene.

MSB agar is used for the isolation of *S.mutans*. MSB agar induce growth of *S.mutans* by provisioning bacteria with sucrose as a unique source for carbon, as well 0.2 unit/ ml bacitracin which is added to medium making it highly selective for very small numbers of *S.mutans* from clinical samples. Colonies of *S.mutans* appear small pale-blue granular colonies on MSB medium.

Data demonstrated that 33 (41*%) S.mutans* isolates were isolated from 80 dental plaque individuals age (7-15 years), this result is consistent with the study by Zainb, (2005) in AL-Hilla city he mentioned that *S.mutans* (40%) from dental caries. Also these results correspond to those previously by Israa and Mahdi (2015) in Kufa city, whereas they find that the ratio of *S.mutans* in dental caries patients is (40%).

EL-Sherbiny, (2014) studied dental caries in Egypt, whereas he mentioned that *S.mutans* account at (40%) from this disease.

AL-Jumaily *et al*., (2014) reported that *S.mutans* ration in dental caries cases is (41%) 12 isolates of 29 samples.

It is clear that there is a convergence between the percentages of *S.mutans* in the current study and its proportion in previous studies, this may be due to similarity in the method and conditions of isolation of these bacteria the previous studies which referred to above used the same medium (MSB agar), as well as the method of sampling did not differ significantly from our study.

The quantity, quality, and frequency of sugar intake (e.g. dessert and sugar contain drinks) have a definitive influence on the incidence and prevalence of caries.(Lee, 2013). Also of the other reason leading to the accurse of decay lack of oral hygiene, where the remain the diet in the moth or between the teeth, as well as, not to remove plaque from teeth surface, all of these factors assist in the events the dental caries.

Napimoga *et al.*, (2005),defined a dental caries as a transmissible infections disease, therefore due to transmission bacteria (*S.mutans*) form mother or people relevant to children will gave chance to these bacteria for cause early decay and clear necrosis in your teeth, for this, these teeth are sensitive and weak at aging. Whereas, this phenomenon occur frequently in families with a low income(Li *et al*., 2007; Folayan *et al*., 2015)

Depending on molecular diagnostic methods, an amplification of 16S rRNA from 33 isolates was performed to confirm bacterial identification. The result showed that 28/33 (84.8) of *S.mutans* had 16S rRNA gene with band 507 bp.

Adhraa *et al*., (2016) have mentioned that 22 (100%) isolates are identified as *S.mutans* by 16S rRNA gene.

The classical microbiological cultures methods limits the studies about the identification of specific populations of *S.mutans*, due to over 100 recognized species in the genus *Streptococcus* .Therefore, identification of *S.mutans* isolates by using 16S rRNA is more accurate than bacteriological and biochemical assays. Rampini *et al*., (2011) demonstrate that 16S rRNA gene PCR was sensitivity, specific, and used for diagnosis of culture negative bacterial infections also useful for identification of bacterial pathogens in patients pretreated with antibiotics. However, the conventional methods for identification of microorganisms are sometimes inaccurate. These kinds of problems could be solved by use of our present PCR method, which is simpler, more rapid, species-specific and accurate for identification of cariogenic species. Therefore, our PCR method would facilitate the process of identifying isolates from clinical samples and be more useful than the conventional methods used in previous studies.

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