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Identification of *Streptococcus mutans* from Human Dental Plaque and Dental Caries Using 16SrRNA Gene

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Abstract

One hundred and sixty samples from saliva and dental plaque were sellected from patients with caries active at ages from (4-65) years from Umm Qasr Primary School and Al-Ameria Specialist Dental Center in Baghdad. 15 isolates belong to *Streptococcus mutans* were identified by biochemical tests and Vitek 2 compact system while 22 isolates identified by using Polymerase Chain Reaction (PCR) techniques and sequencing of 16SrRNA with 120 bp by using 16SrRNA the result confermed that these isolates were belong to *S.mutans*.

Keywords: 16SrRNA, *Streptococcus mutans.*

تشخيص بكتريا *Streptococcus mutans* من عينات تسوس الاسنان واللعاب باستخدام جين 16SrRNA

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الخلاصة

جمعت مائة وستون عينة من اللعاب و الاسنان المتسوسة من مرضى يعانون من تسوس الاسنان باعمارمختلفة تراوحت بين (4–65) سنة عند مراجعتهم مركز العامرية التخصصي لطب الاسنان ومن طلبة مدرسة ام قصر الابتدائية ، شخصت خمسة عشر عزلة بانها *S. mutans* بالاختبارات الكيمو حيوية ونظام الفايتك –2 بينما 22 عزلة شخصت باستخدام تقنية سلسلة تفاعل البلمرة الPCR وجين 16SrRNA بحجم120 زوج قاعدة واثبتت النتائج ان هذه العزلات تعود الى *S.mutans* .

Introduction

The levels of mutans streptococci in saliva have been shown to be a means of predicting both caries activity and the transmission risk of the mutans streptococci can be an important etiological factor of caries [1, 2]. However, the quantifcation of mutans streptococci is laborious, and so far levels of mutans streptococci have not been used as an established index of caries diagnosis. The standard medium used for isolating mutans streptococci, Mitis Salivarius Bacitracin (MSB) agar [3], does not have the selectivity that is necessary for morphological discrimination of the colonies to identify the species of mutans streptococci. Development of a practical assay for mutans streptococci is necessary to establish quantifcation of these bacteria as a new index of caries risk. Some laboratories have reported improvements of the MSB medium and have discussed new selective media for mutans streptococci [4, 5].

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Hence, a conventional MSB medium is usually used as the standard method for isolation and quantification mutans streptococci. Polymerase chain reaction (PCR) is a powerful tool for the detection and quantifcation of bacteria. Real-timePCR techniques are increasingly used in diagnosis, especially for anaerobic bacteria and viruses [6]. PCR assays for detecting mutans streptococci are more specifc than conventional culture methods [7, 8].

Oral streptococci, e.g. *Streptococcus mutans* and *Streptococcus sobrinus*, are important constituents of dental plaque. Being able to distinguish between them is believed to be useful for the detection and prevention of dental caries. Biochemical tests and immunological and genetic methods have been used to differentiate them [9, 10]. Because of its high specificity and sensitivity, PCR is currently being applied in a wide range of medical diagnostics and research. The occurrence of several gene copies of 16S rRNA in the cell and the key role of this genetic target for bacterial taxonomy has made it an established target for PCR detection of bacteria in all different fields of microbiology.

Other targets often used for the detection of bacteria are virulence factors, which are species-specific and provide an additional detection marker in order to avoid ambiguous PCR results caused by the high similarity of species-specific rRNA gene sequences, e.g. in oral streptococci. Species specific primers based on the 16S rRNA gene sequences were used for the detection of *S. sobrinus* and *S. mutans* in a direct PCR [11]. The *S. mutans*-specific primers for the 16S rRNA gene were also used in a nested PCR to detect *S. mutans* in dental plaque [12].

Thus, the aim of this study was to assess the effectiveness of PCR detection method in distinguishing *S.mutans* from other oral streptococci. Therefore, we compared the PCR detection method described above with other biochemical test to distinguish *S. mutans* from109 different streptococcal strains

Materials and Methods

Isolation and culture of *Streptococcus mutans*

One hundred and sixty dental plaque and saliva samples were collected from individuals aged from 4 to 65 years old from Umm Qasr Primary School, and Al-Ameria Specialist Dental Center in Baghdad.. Fifteen mutans *streptococci* local isolates were isolated from human dental plaque and dental caries by growing on Mitis Salivarius Agar (MSA), Mitis Salivarius Agar with 200 I.U/L bacitracin (MSBA) and trypticase, yeast, cystine, sucrose agar (TYC) trypticase, yeast, cystine, sucrose, bacitracin agar (TYCSB) and identified according to biochemical test by growing blood agar medium and incubated anaerobically at 37^{o}C for 48 hrs and examined for ability to ferment sucrose, mannitol , sorbitol , melibiose and raffinose ,then confermed by Vitek 2 compact system.

Polymerase chain reaction

Polymerase chain reaction (PCR) was used to confirm the presence or absence of the 16SrRNA genes in the 15 isolates. One colony of each bacterium from an agar plate was used as the template. The DNA was extracted by using ExiPrep^{TM}Plus Bacteria Genomic DNA Kit (BIONEER, Korea) according to protocol for DNA extraction using ExiPrep$^{TM\ 16}$ Plus machine. All primers used in detection *gtf* genes were designed according to Bioedit program and NCBI BLAST http://www.ncbi.nlm.nih. govwebsite with conserved region (80_530). Forward primer GCGACGATACATAGCCGACC and Reverse primer CTCGGTCAGACTTTCGTCCA. PCR was performed with 2 (100 ng) of template DNA in a total reaction volume of 20 µl consisting of 10 µl of GoTaq Green Master Mix (promega), 2 µl of Forword Primer (10 µM) , 2 µl Reverse Primer (10 µM), 4 µl Nuclease free water. The PCR program consisted of 30 cycles of denaturation (94°C for 1 min), annealing (55 and 60°C for 1 min), and extension (72°C for 1 min) and a final extension step at 72°C for 3 min to amplified 16SrRNA. An Master thermocycler gradient (Eppendorf, Germany) was used for PCR. The positive result of 16S rRNA gene was confirmed by 2% agarose gel electrophoresis stained with ethidium bromide, electrophoresed in 75 volt for 1 hr, and photographed under ultraviolet (UV) transilluminator.

Results and Discussion

One hundred and sixty two samples from patients with caries active and dental plaque were culture on selective medium to detect oral *Streptococcus* species Table-1.

Table 1-Number and percentage of samples from patients with caries active and dental plaque according to gender and age

Gender	Age	Sample No.	Percentage
Male	4-12	63	39.3 %
Male	25-65	3	1.87 %
Female	4-12	79	49.3 %
Female	20-65	15	9.3 %
Total		160	100%

In Table-1, we present new data for observed caries rates among the patients with caries active and dental plaque aged from 4-65 years old, higher caries rates were found more often among females than males. The data show that: in female children caries rates are greater than, or equal to male, rates, mature adults typically exhibit higher caries rates in females; a male gender bias in adults is rare.

Ferraro and Vieira [13] examining factors which contributes to caries and how the factor differs in men and women, there results demonstrate that caries risk factors for women include a different salivary composition and flow rate, hormonal fluctuations, dietary habits, genetic variations, and particular social roles among their family.

The isolated bacteria were identified depending on the morphological and microscopic examinations as well as biochemical properties, The VITEK 2 microbial identification system and GP card available was used for the automated identification of *S.mutans*.[14]. The morphological examination of colonies of examined oral *Streptococcus* that grown on MSA and MSBA, TYC and TYCSB indicated that 51 (31.87%) of isolates cannot grow on MSA, MSBA, TYC and TYCSB this result indicated that these isolates are non-oral streptococci while 109 (68.12%) isolates represented oral *Streptococci* Figure-1.

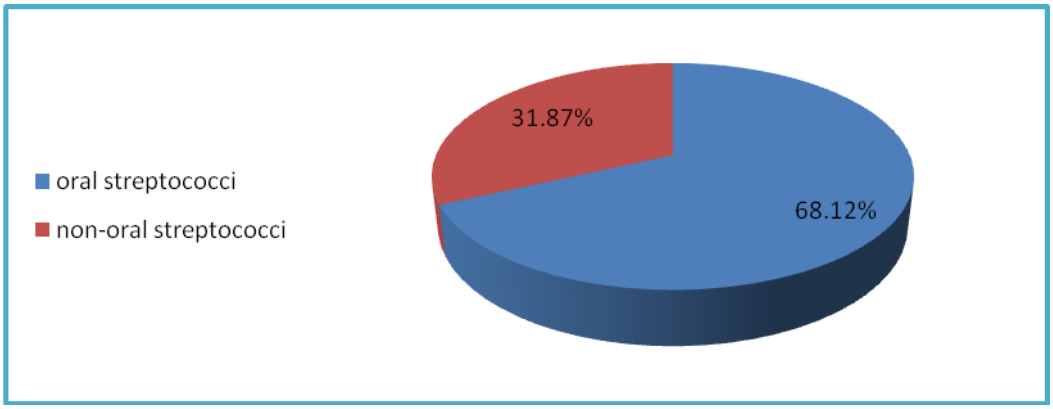

Figure 1- Oral Streptococci isolated from patient with caries active

From 109 oral Streptococci, 22 isolates of *S. mutans* exhibited gamma hemolysin on blood agar, the colonies form rough, heaped, irregular colonies, resembling frosted glass, mostly crumbly, whole colonies can be picked of the agar. White or gray in color on TYCSB agar , colonies are 0.5–2.0 mm in diameter and may have a drop of liquid (water-soluble glucan) on top or a puddle of polysaccharide around the colony on MSBA Figure-2(A, B).

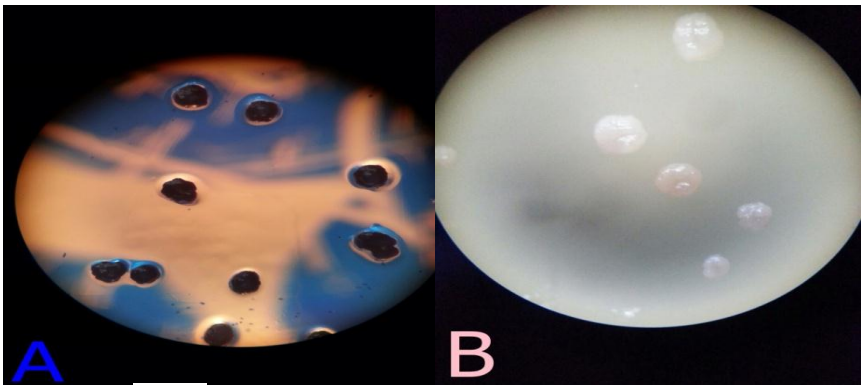

Figure 2- Colonies of S. *mutans* isolates under dissecting microscope 4X A: growth on MSBA; B: on TYCSB

In addition, the isolates were able to ferment mannitol, sorbitol, melibiose, raffinose and mannitol with bacitracin and showed negative result for production catalase and hydrolysis of arginine Table-2. Moreover, Vitek 2 compact system was used for confirmation the identification of *S. mutans* isolates and showed 15 isolates belong to *S. mutans* and gave 89-91% similarity.

Table 2- Colonies characteristics and biochemical tests of 22 *S.mutans* isolates.

Biochemical test		Results
Colony color after sprayed with TTC on MSBA		100% Pink colonies
Blood agar		100% (γ-hemolysis)
Hydrolysis of arginine (NH3) production		100% (-)
Gram stain		100% + ve
Ability to ferment Carbohydrates and produce acid	Mannitol	100% yellow color (+)
	Sorbitol	100% yellow color (+)
	Raffinose	100% yellow color (+)
	Melibiose	100% yellow color (+)
	Mannitol+ Bacitracin	100% yellow color (+)

(+): positive; (-): Negative

Polymerase Chain Reaction (PCR) Techniques

An amplification of 16S rRNA from 22 isolates was performed to confirm bacterial identification. Primers for conserved region of 16S rRNA were designed and used for amplification of DNA of *S. mutans* isolates by PCR then PCR products were separated on agarose gel Figure-3. The result demonstrated that 22 (100%) of *S. mutans* had 16S rRNA gene band with 120 bp. Identification of *S. mutans* isolates by using 16S rRNA is more accurate than bacteriological and biochemical assays.

Rampini *et al* [15] demonstrate that 16S rRNA gene PCR was sensitivit, specific,and used for diagnosis of culture-negative bacterial Infections also useful for identification of bacterial pathogens in patients pretreated with antibiotics.

The PCR products of isolate were submitted to Macrogen Company for sequencing. Sequences were edited using Bioedite sequences software and compared with sequences reported in GeneBank (National Center for Biotechnology Information NCBI). The isolate Sm15 was showed 100% similarity to *S. mutans*.

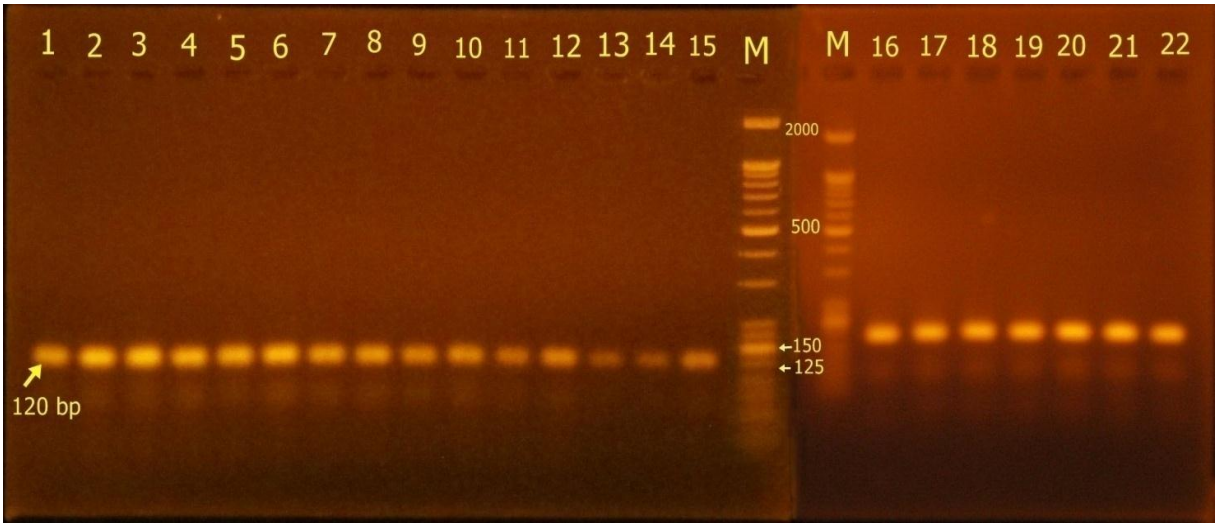

Figure 3-Amlification of a 120-bp 16s rRNA gene of *S.mutans* isolates on agarose gel (2%) electrophoresed in 75 volt for 1 hr, M: molecular marker (25bp DNA Ladder), lanes 1 - 22 refer to (Sm1-m12).

The PCR product was sent to Macrogen Company in South Korea for sequencing of 16SrRNA. The sequencing result of 16SrRNA shown in Figure-4, 91 nucleotides sequence was obtained that cover part of *S. mutans* 16SrRNA gene that composed of 1552 nucleotide.

CCAAGTGCGA CGATACATAG CCGACCTGAG AGGGTGATCG GCCACACTGG GACTGAGACA CGGCCCAGAC TCCTACGGAG GCAGCAGTAG G.

Figure 4- Sequencing result for 16SrRNA primer set by Macrogen Company.

The sequence was blasted in NCBI against standard strain of *S. mutans* complete genome. The identifying result showed 100%, Figure-5. PCR methods are simple and rapid Sato *et al* [12] used two PCR methods to saliva and native plaque substances to distinguish between *S. mutans* and *S. sobrinus*, the most frequently detected cariogenic oral streptococci with two set of primers one for 16S rRNA gene and the other for *gtfB* gene . Al-Ahmad *et al* [16] identified *S. mutans*-specific primers used in nested 16S rRNA gene PCR were not specific for *S. mutans*, but also detected 12 other tested streptococcal strains, including important oral streptococci. In the present study, the *S. mutans*-specific primers used in 16S rRNA gene PCR were specific for *S.mutans* and no need to another primer for another gene to detected cariogenic *S. mutans.*

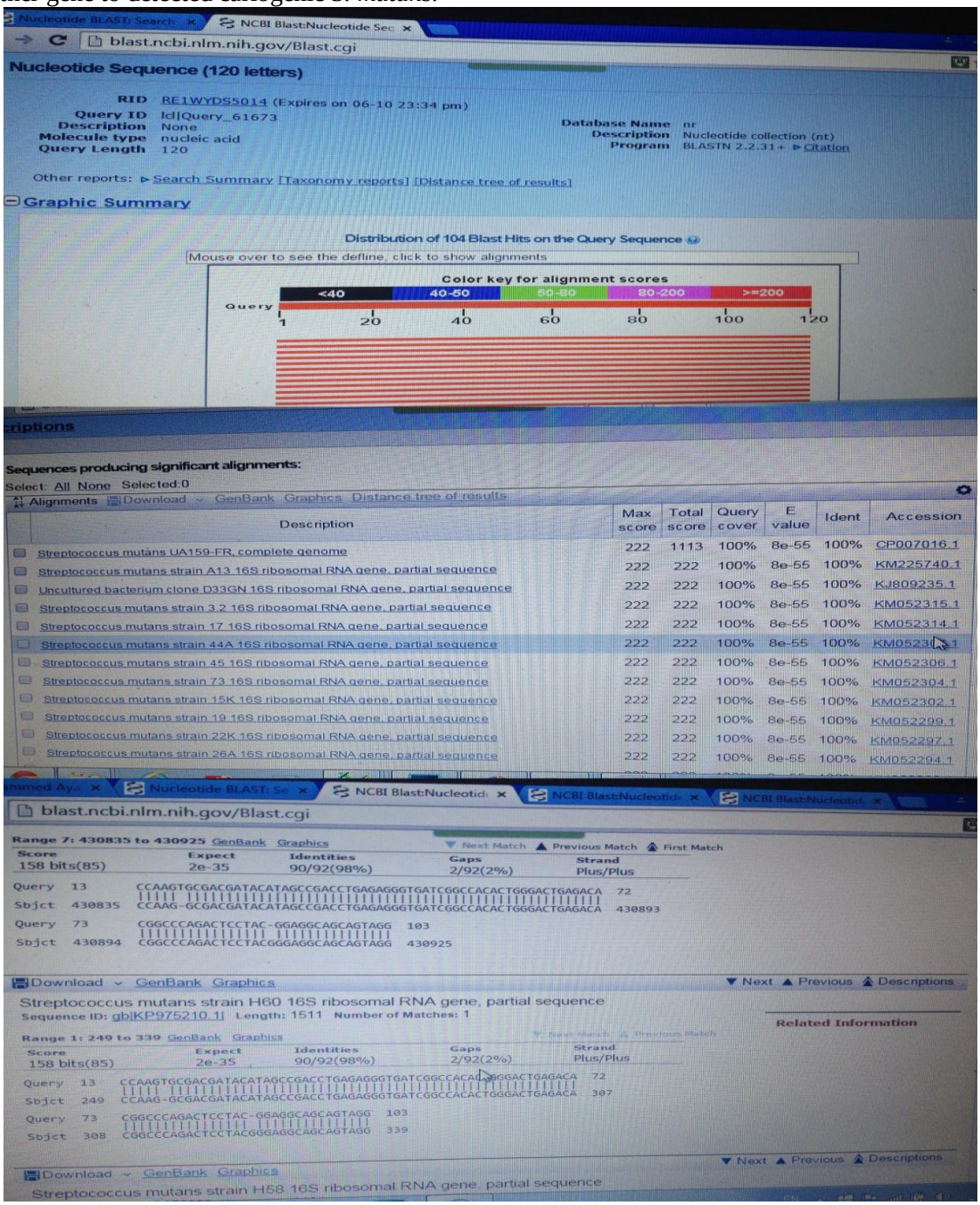

Figure 5- Sequencing of 16SrRNA primer, the sequence was blasted in NCBI against standard strain of *S. mutans.*

References

1. Klock, B. and Krasse, B. **1979**. A comparison between different methods for prediction of caries activity. *Scand J Dent Res*. 87, pp:129-139.
2. Bhalla, S., Tandon, S. and Satyamoorthy, K. **2010**. Salivary proteins and early childhood caries: a gel electrophoretic analysis. *Contemp Clin Dent*, 1(1), pp:17–22.
3. Whiley R. A. and Hardie, J.M. **2009**. *The Genus Streptococcus* In: *Bergy's manual of systemic bacteriology*. Second Edition. De Vos, P., Garrity, G. M., Jones, D., Krieg, N. R., Ludwig, W., Rainey, F. A., Schleifer, K., Whitman, W. B. eds. Springer: USA. 3, pp:655–711.
4. Kimmel, L. and Tinano, N. **1991**. A modified mitis salivarius medium for a caries diagnostic test. *Oral Microbiol. Immunol*. 6, pp:275-279.
5. Wan, A.K., W.K. Seow, L.J. Walsh and P.S. Bird. **2002**. Comparison of five selective media for the growth and enumeration of *Streptococcus mutans*. *Aust Dent J*, 47, pp:21-26.
6. Sakamoto, M., Takeuchi, Y., Umeda, M., Ishikawa, I. and Benno, Y. **2001**. Rapid detection and quantification of five periodontopathic bacteria by real-time PCR. *Microbiol. Immunol*. 45, pp: 39-44.
7. Ono, T., Hirota, K., Nemoto, K., Fernandez, E.J., Ota, F. and Fukui, K. **1994**. Detection of *Streptococcus mutans* by PCR amplification of spaP gene. *J Med Microbiol*. 41, pp:231-235.
8. Igarashi, T., Yamamoto, A. and Goto, N. **1996**. Rapid identification of mutans streptococcal species. *Microbiol. Immunol*. 40, pp:867-871.
9. Beighton, D., Russell, R. R. B. and Whiley, R. A. **1991**. A simple biochemical scheme for the differentiation of *Streptococcus mutans* and *Steptococcus sobrinus*. *Caries Res* 25, pp:174–178.
10. Cangelosi, G. A., Iversen, J. M., Zuo, Y., Oswald, T. K. and Lamont, R. J. **1994**. Oligonucleotide probes for mutans streptococci. *Mol Cell Probes* 8, pp:73–80.
11. Rupf, S., Merte, K., Eschrich, K., Sto¨ sser, L. and Kneist, S. **2001**. Peroxidase reaction as a parameter for discrimination of *Streptococcus mutans* and *Streptococcus sobrinus*. *Caries Res* 35, pp:258–264.
12. Sato, T., Matsuyama, J., Kumagai, T., Mayanagi, G., Yamaura, M., Washio, J. and Takahashi, N. **2003**. Nested PCR for detection of mutans streptococci in dental plaque. *Lett Appl Microbiol* 37, pp: 66–69.
13. Ferraro, M. and Vieira, A. R. **2010**. Explaining Gender Differences in Caries: A Multifactorial Approach to a Multifactorial Disease. *Inter J of Dent*. Article ID 649643, 2010, 5 pages.
14. BioMerieux, S.A. **2010**. VITEK 2 systems product information. Durham, North Carolina.
15. Rampini, S. K., Bloemberg, G. V., Keller, P.M., BÜchler, A. C., Dollenmaier ,G. Speck, R. F., and BÖttger, E. C.**2011**. Broad-Range 16S rRNA Gene Polymerase Chain Reaction for Diagnosis of Culture-Negative Bacterial Infections. *Clin Infect Dis*.53(12), pp:1245–51
16. Al-Ahmad, A., Auschill, T. M., Braun, G., Hellwig, E., and Arweiler, N. B.**2006**. Overestimation of *Streptococcus mutans* prevalence by nested PCR detection of the 16S rRNA gene. *J. of Med Microbiology*, 55, pp: 109–113.