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Comparison of Smoker and non-Smoker Gingival Microbial Population and Identify the More resistant Isolates by 16SrRNA

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Abstract: *Fifty gingiva swab samples were collected from males, 25 smokers and 25non-smokers. The samples were collected and analyzed at Baghdad University .Ggram positive bacteria belong into two genus;Streptococcusand Staphylococcus(aureus and epidermidis) were isolated, while E. coli andKlebsiella represent the isolated Gram negative genus.Streptococcus appears in 44% and 64% of non-smoker and smoker sample,respectively. Staphylococcus aureus appear in higher frequency in smoker(24%) than non-smoker (4%) in contrast, Staphylococcus epidermidis compromise 36% of non smoker samples and 0% in smoker samples, in general gram negative bacteria appear in lower proportion in both smoker and non-smoker samples, no gram negative bacteria appear in smoker samples comparing to 8% of E. coli and Klebsiellain non-smoker samples. The bacterial identification is confirmed for Streptococcus mutans, as it is the most frequent bacteria was isolated, by using of PCR and sequencing for 16S rRNA. The results of sequencing revealed that the streptococcus mutansisolated is this study; appeared 98% compatibility with reference. The score revealed high similarity toStreptococcus mutans gene for 16S ribosomal RNA, partial sequence, strain: JCM 5175. GenBank: LC311064.1.In addition the phylogenetic tree shows similarity to Saudia Arabia which is acceptable as the two countries are in contact continually.*

Key wards: *Smoker, non-Smoker, Gingival, 16S rRNA.*

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I. Introduction

The Gum disease is an infection of the gums that affect the bone structure which supports the teeth. This infection can lead to teeth fall out in severe cases. The important cause of severe gum disease is smoking in the U.S. [1].Gum disease starts with bacteria on teeth that get under the gums. If germs stay on teeth for long time, layers of plaque and tartar develop which leads to gingivitis [2]. Smoking is the practice of which a substance is burned, resulting smoke thatinhaled to be tasted then it will be absorbed into the bloodstream. Smokingis a route of administration of drug use to combustion of dried plant leaves which vaporizes and delivers active substances into the lungs where they can be rapidly absorbed into the bloodstream and reach tissues [3].Smokingis an important cause of lung cancer and also cardiovascular disease. Moreover it may cause other diseases in the mouth. Also the dental implant may be fail among the smokers than among non-smokers, gum disease can occur around these[4].

Many oral diseases are associated with smoking like, Staining of teeth, Lowing of the ability to smell and taste, Bad breath, Smoker's palate, in which the palate turned to white with spots project from the surface bearing a red spot in theircenterwhich marks the duct opening of the gland,melanosis, that associated with cigarette and can be seen as brown spots inside the smoker mouth. Moreover smoking can cause coated tongue, a condition of forming colored layer which composed of food particles, bacteria and debris from epithelial cells in the mouth.Also oral thrush caused by fungal infection andoral precancer and cancer. These conditions may be result from the Irritants, toxic and cancer compounds that found in the smoke. Thedryness in the mouth followed by high temperatures of inhaling smoke with pH change effect on immune response and susceptible to viral and fungal infections [5,6]. Moreover antibiotics effect on many types of this flora could be less with the time due to their resistance [7]. Chronic gingival disease occurring mostly in adults, but it can be seen in younger people. Destruction is related to the amount of plaque present, also other local factors[8]. In a study, the prevalence of bony lesions is indicative with chronic inflammatory periodontal disease, 0.5%[9]. The gingival tissues respond is occur within 4 days to a beginning accumlation of microbial plaque [10].

II. Materials and methods

Sample collection

Fifty swab samples were collected from male gingiva, 25 samples from smokers and 25 from non-smokers at Baghdad University were screened.The samples were collected and analyzed according to the method of Gholanireza*et al*. 1992, .using streaking plate technique. The person was first washed his mouth, then a sterile swab was rotated over the surface of his gingival at all sides.

Samples culture

The gingival swabs were streaked onto blood agar and preserved in nutrient broth. The plates and tubes were incubated aerobically at 37 °C for 48 hours. Also, the swabs were streaked onto MacConky agar and the plates were then observed for the presence of isolated colonies after overnight incubation. The isolated microorganisms were transferred from the petri plate to a tube containing the nutrient agar (slant).After this, pure cultures of bacterial isolates were characterized based on morphological and biochemical tests.Bergy's manual of systematic bacteriology was used as reference for identification.

PCR test

DNA Extraction: Bacterial samples of overnight culture were used for DNA extraction, bacterial DNA was extracted from the bacterial culture using G- spin DNA extraction kit, intron biotechnology, cat.no. 17045. The extraction was done according to the kit protocol. Primers were ordered from Integrated DNA technologies /USA.Specimens were processed PCR assayusing specific primers of 16S RNA of gene, Forward5'- AGAGTTTGATCCTGGCTCAG- 3', Reverse 5'- GGTTACCTTGTTACGACTT- 3'. The optimal condition has identified for Initial denaturation and annealing temperature after several experiments using Gradient PCR for all samples and also changed the concentration for DNA template between (1.5-2µl).

Maxime PCR PreMix kit (i-Taq) 20µlrxn (Cat. No. 25025)

iNtRON's*Maxime*PCRPreMixKit has not only various kinds of PreMixKitaccording to experience purpose, but also a 2X Master mix solution. *Maxime*PCRPreMixKit (*i*-Taq) is the product what is mixed rxnPCR to get the best result.

Diagnosis of Gene:

Table1: Mixture of the specific interaction for diagnosis gene

Components Concentrations of the test mixture were,Taq PCR PreMix (5µl), primers (10 picomols/µl) from each one, DNAtemplet (1.5µl), Distill water (16.5 µl), the final volume was 25µl.

Table1: The optimum condition of detection gene

No.	Phase	Tm (°C)	Time	No. of cycle
1-	Initial Denaturation	95°C	3 min.	40 cycle
2-	Denaturation -2	95°C	45sec	
3-	Annealing	52°C	45sec	
4-	Extension-1	72°C	50sec	
5-	Extension -2	72°C	10 min.	

Gradient Annealing: 52, 54, 56, 58, 60, 62.

Red safe Nucleic acid staining solution

RedSafe Nucleic Acid Staining Solution (20,000x); anew and safe nucleic acid stain for detecting nucleic acid in agarose gels.

Prepare of the Agarose gel

The agarose gel was preparedaccording to Sambrook*et al*,.1989, the agarose gel has been made in 1% and 2% condensation by melting (1, 2) g of agarose in 100 ml of previously made TBE buffer.

Sensitivity test

An amount of 0.1 ml broth was taken and put in tube, diluted with normal saline andwas compared with Makfarland. Several dilutions were done to avoid heavy growth on Muellarhinton.Antibiotic discks were put,Vancomycin and Cefatrixen for G +ve, Ogmentin and Cloxacillin for G –ve. The plates were incubated for 24 hr then the diameter of the inhibition zone was measured.

Sequencing for *Streptococcus mutans* PCR product

The samples were sent to Microgen/koria, the gene sequencing process was done using genetic analyzer (Applied Biosystem) and homologysearch wasperformed using (BLAST) program online using blastn and blastx algorithms which are available at NCBI

Result and discussion

The culture results of the collected samplesrevealed that the bacteria were belong to four strains that are:G –ve (*E.coli* and *Klebsiella*), G +ve (*Staphylococcus* and *Streptococcus*) as shows in table 1 Streptococcus was isolated from 64% of smoker persons and its higher percent comparing to the non-smoker person (44%).

Table 2:comparation between smoker and non smoker bacterial culture result

	Non Smoker			**Smoker**	
Streptococcus	**11**	**44%**		**18**	**64%**
Staphylococcus aureus	**1**	**4%**		**6**	**24%**
Staphylococcus epidermidis	**9**	**36%**		**0**	**0%**
E. coli	**2**	**8%**		**0**	**0%**
Klebsiella	**2**	**8%**		**0**	**0%**
No growth	**0**	**0%**		**0**	**0%**

Twenty five species of streptococci may be live and colonize in theoral cavity. Each onecandeveloped specific characteristics for colonizing in different oral site and can cause changing inoral environment conditions in order to competethe other types of bacteria and withstand the external challenges. The diseases in oral cavity, can initiate as a result of Imbalances in the microbial biota. The commensal streptococci may switch to opportunistic pathogens under special conditions in oral cavity leading toinitiate the disease which can cause severe damage.For example oral streptococci are both harmless and harmful bacteria. *StreptococcusMutans* isone of the most important bacteria that associated with tooth decay and carious lesions [12]. one study in showed that *S. mutans* is more common on the pits and fissures, in proportion of 39% of the total streptococci in human oral cavity but, it is lower in the buccal surface (2–9)% [13].Manyresearchs indicates that *S. mutans*is themost type of bacteria in smokers that cause gum disease. Smoking alters the body immune response to the bacteria that found in plaque. It decreases the ability of the body immune response to the bacteria and thus causes the disease.Many compounds in smoke constitute can reduce in the immune system, especially nicotine. Inflammation is the main way of our body to respond to bacteria, neutrophils are the most importantcellsedinvolved in protection against gum disease. The numbers of neutrophils in smokers are more non-smoker that of no in the body in total; but fewer neutrophils can reach the gums due to the effects of nicotine. As a result,neutrophils cannot control the bacteria leading to increase the chance for gum disease to occur. Moreover,in smokers the destruction of the gums is faster because of the higher number of matrix metalloproteinases, interleukin-1, prostanglandin-2. These are immune response components of the body which are involved in inflammation process. Nicotine and other compounds in tobacco may nave detrimental effects on the blood system, inflammatorion and immune system. The smoking has an effect on the staphylococcus species of isolated from gingival, 24% of smoker were carrying *Staphylococcus aureus* 6 times more than the non-smoker. O-An opposite result was obtained with *Staphylococcus epidermidis*, 36% of non-smoker was carrying this bacteria and no one from smoker was detected as carrying the bacteria [12].Staphylococci are recognized as constituents of the normal oral flora [14].

The bacterial isolation in this study are 1 (4%) isolate of *Staphylococcus aureus*, 9 (36%) of, *E. coli* 2 (8%), 2(8%) of *Klebsiella* from smokers and 81(64%) of *Staphylococcus aureus*, 6 (24%) of *Staphylococcus aureus* from non-smoking. In a previous study the isolation rates for Staphylococcus aureus according to the population studied, the reported rates were 24%-84% in healthy adult dentate oral cavities[15,16] and an incidence of 48% among the denture-wearing population.[17]. In addition, it has also been reported that *S.aureus* may have a role in dental implant failure [18,19]. It seems likely that in line with infections caused by *S.aureus* at other body sites, a number of oral staphylococcal infections may be the result of cross-infection from different sources[20].

The presence of the rest bacterial species (*E. coli* and Klebsiella) can be accidental during the work or from some contaminated food or during culturing.

Antibiotic Sensitivity Results

Generally, a G+ vebacteria seems more sensitive than G-ve bacteria. The results show that Streptococcus is more sensetive to Cefitriaxon than with Vancomycin, but the same effect is seen of the two antibiotics against Staphylococcus. Augmentin and Cloxacillin have the same effect approximatly on *E. coli* and klebsiella.

This study revealed that *E. coli* and *Klebsiella* have shown similar pattern of sensitivity to augmentine, 20mm and 22mm respectively, and for the Cloxacillin the results of inhibition zones are 18mm and 20mm respectively.

Table3:Inhibition zone measured in mm on Muller Hinton agar.

	Cefitriaxon	Vancomycin	Augmentin	Cloxacillin
Staphylococcus	**31 mm**	**30 mm**	**-**	**-**
Streptococcus	**40mm**	**22mm**	**-**	**-**
E.coli	**-**	**-**	**20mm**	**18mm**
Klebsiella	**-**	**-**	**22mm**	**20mm**

Staphylococcus and Streptococcus sensitivity to antibiotics were to Cefitriaxon and Vancomycin. The inhibition zone of cefitriiaxon is 31mm ad 40 for Staphylococcus and Streptococcus respectively and the inhibition zone of Vancomycin is 40mm, 22mm for Staphylococcus and Streptococcus respectively.

An important fact that has to be realized is bacterial resistance to antibiotics which has been developing along with every discovery of new antibiotics. Many factors are involved and even in the most developed countries this problem is present. The pathogens have fought for their survival and newer mutant strains had developed, thus making this problem more difficult to control the infection. The discovery of novel antibiotics may be taken a slower pace as compared to the emerging lethal strains, despite, the advanced researches that gives these pathogens an edge to our species [21]. For that reason, a very targeted treatment is necessary for control these infections and to prevent antibiotic resistance. The cost effectiveness of the antibiotics needed to treat these drug resistant is another issue which has to be dealt with as this would be very expensive [22].

PCR Results

The results of PCR for detection the bacterial isolates are confirmation of isolalates identification,*Staphylococcus aureus*, *Staphylococcus epidermitis*, *Streptococcus mutans*, *Klebsiella*but for *E. coli*ther is no 16S RNA gene product, the results are demonstrated in table 6. In the recent years, several culture-independent techniques, like checkerboard DNA–DNA hybridization, or polymerase chain reaction (PCR), *in situ* hybridization have been developed to overcome the low sensitivityofcultivation [23]. The analysis of the microbiological diagnostic methods suggests that PCR is the most appropriate approach for the identification of certain pathogens, as it is a better sensitivity and the *in situ* hybridization is applied more in research to solve specific scientific tasks [24].

Specific genes, such as 16S rRNA genes, contain specific signature sequences to anorganisms of the same species.But, PCR method does not provide information about the bacterial morphology, number and the microorganism cellular environment [25]. PCR method involves an amplification of a region of DNA flanked by selected specific primers for the target species [26]. The presence of the amplification product revealed the presence of the microorganism. Among different detection methods, PCR displays the best detection method, identifying a few cells and it shows no cross-reactivity [27].

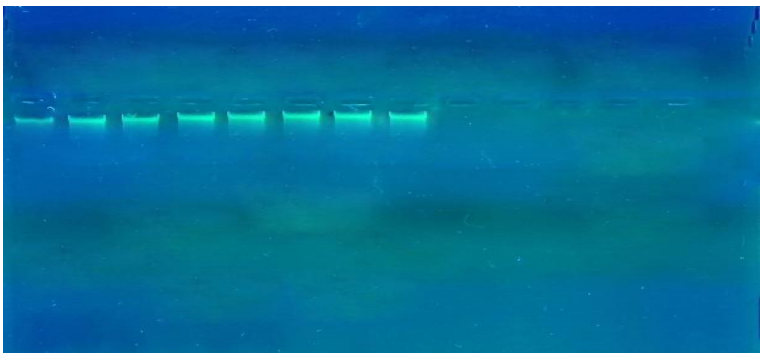

Figure 1: Gel electrophoresis of genomic DNA extraction from bacteria, 1% agarose gel at 5 vol /cm for 1:15 houre.

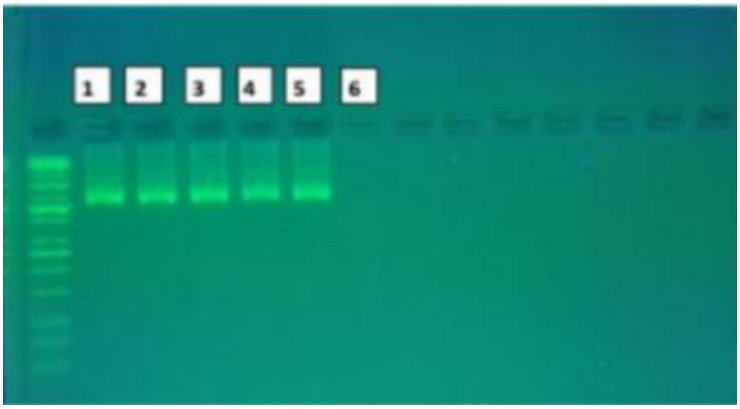

Figure 2: PCR product the band size 1250 bp. The product was electrophoresis on 2% agarose at 5volt/cm^2. 1x TBE buffer for 1:30 hours. N: DNA ladder (100) (KAPA Universal DNA Ladder (cat # KK6302)
1: Positive control, 2: Streptococcus mutans, 3: Klebsiella, 4, S.epi, 5: S.aureus 6:Ecoli

Table 4: PCR Results:

Sample	Name of Sample	DNA Result	PCR Result 16srRNA
1	***Streptococcus mutans***	+	+
2	***Klebsiella***	+	+
3	***S.epi***	+	+
4	***S.aureus***	+	+
5	***Ecoli***	+	-

The existing data in the literature confirms our results for the better diagnostic capability of the PCR method. Furthermore, our data demonstrate that current targets, for *E.coli*, are not suitable as species-specific genes and that may be due to sequence variation.

Nucleic acid sequencing was done to the PCR product of *Streptococcus mutans* 16S rRNA partial gene as study this bacteria was the most predominant isolate to confirm the detection of this bacteria. In this study, local isolate was analyzed and compared with the reference strain available in the Genbank database national center for biotechnology information (NCBI). After sequencing results appeared 98% compatibility with reference. The score revealed high similarity to*Streptococcus mutans* gene for 16S ribosomal RNA, partial sequence, strain: JCM 5175. GenBank: LC311064.1.

Tabla : Sequencing ID in GenBank, score, Expect and compatibility of sequences for
Tabke5: *Streptococcus mutans* partial 16S rRNA gene

Type of substitution	Location	Nucleotide	Range of nucleotide	Sequence ID	Score	Expect	Identities	SOURCE
Trinsvertion	**556**	**C>G**	64 to 820	ID: AJ554208.1	1306	0.0	98%	Streptococcus mutans
Trinsvertion	**596**	**G>C**						
Trinsvertion	**700**	**T>A**						
Transition	**712**	**G>A**						
Transition	**717**	**A>G**						
Transition	**728**	**G>A**						
Trinsvertion	**738**	**G>T**						
Trinsvertion	**761**	**G>T**						
Trinsvertion	**765**	**T>G**						
Trinsvertion	**777**	**T>A**						
Transition	**798**	**G>A**						
Trinsvertion	**808**	**A>T**						
Trinsvertion	**817**	**C>A**						

Streptococcus mutans partial 16S rRNA gene, strain CECT 4034
Sequence ID: AJ554208.1Length: 849Number of Matches: 1
Related Information
Range 1: 64 to 820GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
1306 bits(1448)	0.0	744/757(98%)	0/757(0%)	Plus/Plus

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Query  1    ATTAGCTAGTAGGTAGGGTAACGGCCTACCTAGGCAACGATACATAGCCGACCTGAGAGG
                                    60
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  64   ATTAGCTAGTAGGTAGGGTAACGGCCTACCTAGGCAACGATACATAGCCGACCTGAGAGG
                                    123

Query  61   GTGAACGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGG
                                    120
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  124  GTGAACGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGG
                                    183

Query  121  AATCTTCGGCAATGGACGCAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGACGGTTTTC
                                    180
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  184  AATCTTCGGCAATGGACGCAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGACGGTTTTC
                                    243

Query  181  GGATCGTAAAGCTCTGTTGTAGGGGAAGAACGTGTGTAAGAGTGGAAAGCTTACACAGTG
                                    240
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  244  GGATCGTAAAGCTCTGTTGTAGGGGAAGAACGTGTGTAAGAGTGGAAAGCTTACACAGTG
                                    303

Query  241  ACGGTACCCTACCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG
                                    300
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  304  ACGGTACCCTACCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG
                                    363

Query  301  TCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGGGAGCGCAGGCGGTTTAGTAAGTCTG
                                    360
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  364  TCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGGGAGCGCAGGCGGTTTAGTAAGTCTG
                                    423

Query  361  AAGTTAAAGGCATTGGCTCAACCAATGTATGCTTTGGAAACTGTTAGACTTGAGTGCAGA
                                    420
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  424  AAGTTAAAGGCATTGGCTCAACCAATGTATGCTTTGGAAACTGTTAGACTTGAGTGCAGA
                                    483

Query  421  AGGGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCGG
                                    480
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  484  AGGGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCGG
                                    543

Query  481  TGGCGAAAGCGGGTCTCTGGTCTGTCACTGACGCTGAGGCTCGAAAGCGTGGCTAGCGAA
                                    540
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Sbjct  544  TGGCGAAAGCGGCTCTCTGGTCTGTCACTGACGCTGAGGCTCGAAAGCGTGGGTAGCGAA
                                  603

Query  541  CAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGCTGAGTGCTAGGTGTTAGGTCCTT
                                  600
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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                                  663

Query  601  TCCAGGACTTAGTGCCGACGCTAACGCATTAAGCACACCGCCTGGGGAATACGGCCGCAA
                                  660
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Sbjct  664  TCCAGGACTTAGTGCCGACGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAA
                                  723

Query  661  GGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAATCGGGGGAGCATGTGGATTAATT
                                  720
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  724  GGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT
                                  783

          Query  721  CGAAGCAACGCGAAAAACCTTACCTGGTCTTGAAATC  757
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          Sbjct  784  CGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATC  820
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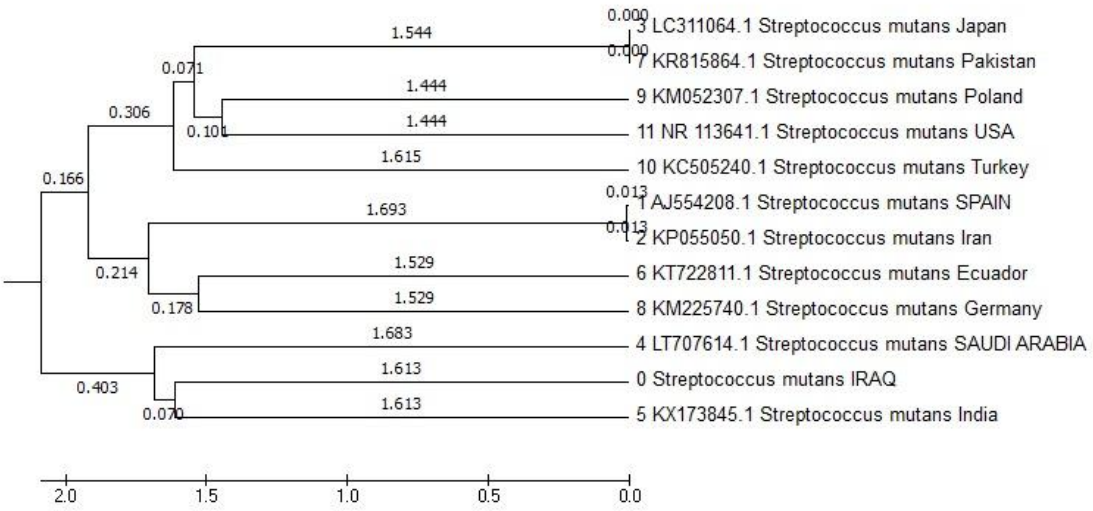

Figure: Evolutionary relationships of local strains of *Streptococcysmutans*as demonstrated as phylogenetic tree.

The sequence results of the isolate of S*tptococcusmutans*in this study show some variation in mutations in 16S rRNA. In addition the phylogenetic tree shows similarity to Saudia Arabia which is acceptable as the two countries are in contact continually.

III. Conclusions

Streptococcus was isolated more frequently from smoker than non-smoker. The current study revealed that Smoking effect on the Staphylococcus species that founded in the gingival culture. Cefitriaxon have the higher effect on Streptococcus.

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