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Phenotypic and Molecular characterization of Streptococcus mutans isolated from Teeth decayed in Babylon Province

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Abstrct

Many strains of S. mutans caused dental plaque patients through the world. Various genotypes of S. mutans have been found in salivation, and dental biofilm. This study conducted in Babylon province middle of Iraq in 2021-2022. Fifty mouth wash samples were collected from patients suffering from severe teeth decay. The aim of study to phenotypic and genotypic the strains of S.mutants. The result shown growth of S.mutans colonies on MSB agar were characterized by special colony morphology, rough, dark blue color. The specific primer pair was successfully amplified partial sequence of dexA gene of 8 colonies pre-identified as S.mutans on phenotypic and biochemical tests. The amplification shown PCR band with size 614bp. Genotyping S.mutans isolates based on Sequence analysis shown nine strains of S.mutans were subjected for sequence analysis; the results of sequence were subjected for multiple alignments by Geneious prime software. Four variants were observed with high frequency, variant 1 wild type T mutant to G (T>G) with frequency 33.4%; variant 2 C>t (44.4%); variant 3 A>C (66.6%) and Variant 4 C>T (44.4%). The Frequency values included homozygous mutation and heterozygous mutation. The final finding that S.mutants undergo high microevolution and produce many genotypes , this genotypes considered as risk factor in disease disappeared and treatment.

Keywords

Streptococcus mutants, phenotypic, genotypes, PCR. dexA gene.

Streptococcus mutans is considered naturally present in the human oral microbiota, along with at least 25 other species of oral streptococci. S. mutans has specific properties for colonizing different oral sites. S. mutans is most prevalent on the pits and fissures, constituting 39% of the total streptococci in the oral cavity. Fewer S. mutans bacteria are found on the buckle surface (2−9%) (Nicolas and Lavoie 2011).

S. mutans strains have been proposed to be implicated in the aggravation of ulcerative_colitis. Such possibilities should not be ruled out without an accurate pathological explanation (Mang-de la Rosa et al., 2014; Belibasakis et al.,2019). Various genotypes of S. mutans have been found in salivation, and dental biofilm. One of these genotyping methods was RAPD-PCR method, has been generally used to separate this genotypic diversity (Arthur et al., 2007).

Oral assessments to decide the DMF-T Index (Decay, Missing and Filling Teeth) were completed. Secludes were distinguished by the Streptoccocus sobrinus and Streptococcus mutans PCR, API 20STREP and 16S rRNA succession examination. Genotypes c, e, f and k were distinguished by PCR. Results: Prevalence of S.

mutans was 14.9%. Of the 47 salivation tests, 57.4% (27) related to youngsters with dental caries, and 8.5% (4) were positive for S. mutans genotype c, 2.1% (1 every) genotype f genotype k, and genotype c and k, individually (Oho et al.,2000).

Napimoga et al. (2004) additionally found a limit of eight genotypes in caries-dynamic subjects utilizing AP-PCR. Nonetheless, it has been seen that youngsters harbor just one to five unmistakable genotypes of S. mutans (Mattos-Graner et al.,2001; Grцnroos and Alaluusua (2000). The consequences of this examination are reliable with past investigations revealed in kids. It was seen that in the sans caries bunch, 10 preschool youngsters had just a single genotype.

Franco et al., (2007) referred to the issue of separation between S. mutans and S. sobrinus is difficult, and is additionally a tedious methodology. In this manner, immunological and biochemical, testing has now been performed on screened bacterial states. All things considered, a polymerase chain response (PCR) strategy for straightforward, fast and dependable distinguishing proof of MS species has likewise been created (Oho et al.,2000; Nakano et al.,2004).

Materials and Methods

Collection of Samples

Fifty mouth wash samples were collected from patients suffering from severe teeth decay. Cases were diagnosed by special physician. A wash samples were collected from Private Clinic in sterile tubes size 10 ml for each patient. Samples were stored in a cool place (4°C) then were transported to the laboratory. The control group was composed of 50 of mouth washes were collected randomly from healthy persons without undergo any teeth decay symptoms. All do not up take any antibiotic before three days ago, both with age ranged (20-60) years during the period from the beginning of Dec.2021 −Jun.2022.

Isolation of Mutans Streptococci

One hundred microliter of undiluted samples was spread on the surface of mitis salivarius agar (MSA) plates using sterile loop. Cultures were incubated anaerobically for 48 h., aerobically overnight at 37°C. Count of more than 250 colonies (104 CFU/ml) was considered as positive samples (Salman et al.,2017). The following methods were used for initial characterization of the isolates: Colonial shape and form on MS-agar and BA, Gram-staining and Microscopically examination., Biochemical test. (Nada et al.,2008).

Phenotypic and biochemical identification of S.mutans

Mitis Salivarius Bacitracin Agar (MSBA) by added (20%) of sucrose and (0.2) g / mL of Bacitracin, which represses the development of most microbes aside from S.mutans and S.sobriuns 20 disengages were gotten from S.mutans microbes. The development of disengages on the MSA medium after brooding in anaerobic conditions at 37 °C for 48 h show Small, high, sporadic states were connected to one another.

Identification of bacteria and by using specific primer- PCR for S.mutans to confirm the bacterial infection. The genotype distributions of genetic variants in S. mutans by used the PCR-sequences analysis polymorphisms for S. mutans based on PCR results. Primers were used in the current study are listed in Table 1.

Table -1-: Primers used in the current study.

Primer	Sequence (5' → 3')	Size	Reference
FDX: RDX:	5 -`CGCAGGTTGAAACAGATGC-3' 5`-TGTCGCTTGGCGAACTTGAT`-3'	615bp	Designed in this study

Genomic DNA Extraction from teeth wash.

For each sample, 1000µl of teeth wash was subjected for centrifugation 8000 RPM for 5mints, discharged the supernatant, the residual transferred in to a 1.5ml micro centrifuge tube containing the Proteinase K (PK) solution 20µl, vortex step and then incubated in water bath at 56°C for 15 minutes. After that adding the content of Eppendorf tube into an empty ReliPrep TM Binding Column. Then centrifuge for 3 minute at13,000rpm speed. Place the column in a clean 1.5 micro centrifuge tube and then adding 80µl Nuclease Free Water to the column with centrifuge for 2 minute at 12000rpm. All samples has been stored at -20 °C for future works.

PCR condition

The genotypes of S.mutans by polymerase chain response (PCR) and sequencing analysis. PCR were performed in a complete volume of 25 µl containing 50 ng genomic DNA, 1.2 µl(10 pmol) of every primer, 12 µl Master mix (Promega Co.) PCR enhancement was performed in a programmable warm cycler angle PCR framework (Labnet USA). The PCR intensification was performed (pre-denaturation at 94 °C for 1 min,

35 cycles denaturation at 94 °C for 30 sec. annealing temp 56 °C for 40 sec., extension at 72 °C for 40 Sec., and final extension for 3 min at 72 °C). The PCR products were electrophoresis by 1.5 % agarose gel pre-staining with Ethidium bromide and afterward sequenced (Macrogen, Korea).

Sequencing analysis

The resolved PCR amplicons were commercially sequenced at the sequencing company (Macrogen Inc. Seoul, South Korea). The sequence results were subjected to pairwise alignment on NCBI for identification. By comparing the observed DNA sequences of local bacterial samples with the retrieved neighboring DNA sequences of the NCBI Blast engine, Chromatograms multiple alignments for all S.mutants sequences were performed based on genius prime software and phylogeny tree was drew.

Rustles and Discussion

Isolation of the mutans streptococci

The result shown growth of S.mutans colonies on MSB agar were characterized by special colony morphology, rough, dark blue color Figure (1). The culturing method on mitis salivarius agar, considered as selective medium for mutans streptococci revealed that many colonies of S. mutans. The bacterium is reported to be isolated only from caries-active sites, while S. mutans has been isolated from both active and noncaries sites.

Thirty eight bacterial colonies were recuperated on MSB from 50patients (matured 20 to 60 years old). To confine the mutans streptococci, 10 colonies were chosen dependent on the province morphology Figure1.

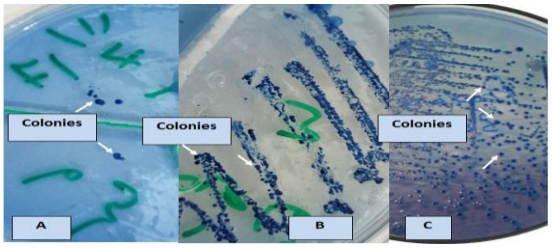

Figure 1: Colonies morphology of *Streptococcus mutans* on mitis salivarius agar plate. A-single colony, B- and C streaking culturing methods and abundant growth.

Molecular identification S.mutans

The specific primer pair was successfully amplified partial sequence of dexA gene of 8 colonies pre-identified as S.mutans on phenotypic and biochemical tests. The amplification shown representative of 8 PCR strains with size 614bp. Figure 2

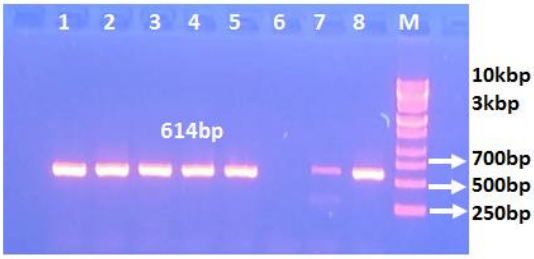

Figure 2: Profile gel electrophoreses of PCR products of amplification partial sequence of dexA gene of S.mutans,The product size was 614bp, wells:1-8 isolates of S.mutans ,M molecular marker 250-10kbp.1.5%agarose, 45min.

Conformation identification of S.mutans by Sequence analysis.

Ten PCR products of S.mutans (WAHEEDA 1 and 9) were selected randomly out of amplification isolates of S.mutans. 18 µl of PCR products were sent to Macrogen Inc., Seoul, Korea for sequencing. The received sequence results shown 9 out 10 came perfect sequence while on become rubbish in sequence. This study was selected the name of WAHEEDA to our S.mutans isolates, based on the personal optional). The nine sequence chart for isolates were alignments with reference sequences deposited in genbank based on the link (https://blast.ncbi.nlm.nih.gov/Blast.cgi), the results of the NCBI report identity percentage 99.35% -99.15% with many reference strains of S.mutans with accession numbers CP01323.1,CP033199.1 and LR13432.1 Figure (3).

Description	Query Cover	E value	Per. Ident	Acc. Len	Accession
Streptococcus mutans strain FDAARGOS_685 chromosome	100%	0.0	99.35%	2054143	CP050962.1
Streptococcus mutans strain P1 chromosome, complete genome	100%	0.0	99.35%	2086371	CP050273.1
Streptococcus mutans strain P6 chromosome, complete genome	100%	0.0	99.35%	2086254	CP050272.1
Streptococcus mutans strain S1 chromosome, complete genome	100%	0.0	99.35%	2080705	CP050271.1
Streptococcus mutans strain S4 chromosome, complete genome	100%	0.0	99.35%	2086365	CP050270.1
Streptococcus mutans strain 35 translation elongation factor ts, I	100%	0.0	99.35%	62987	MK144293.1
Streptococcus mutans NBRC 13955 DNA, complete genome	100%	0.0	99.35%	2018796	AP019720.1
Streptococcus mutans strain NCTC10449 genome assembly, ch	100%	0.0	99.35%	2019343	LS483349.1
Streptococcus mutans strain LAR01 chromosome, complete gen	100%	0.0	99.35%	2068369	CP023477.1
Streptococcus mutans strain FDAARGOS 1458 chromosome, cc	100%	0.0	99.35%	2019345	CP077404.1
Streptococcus mutans strain 27-3 chromosome, complete genor	100%	0.0	99.35%	1978522	CP066294.2
Streptococcus mutans GS-5, complete genome	100%	0.0	99.35%	2027088	CP003686.1
Streptococcus mutans NN2025 DNA, complete genome	100%	0.0	99.35%	2013587	AP010655.1
Streptococcus mutans LJ23 DNA, complete genome	100%	0.0	99.13%	2015626	AP012336.1
Streptococcus mutans strain ATCC 25175 dextranase gene, com	100%	0.0	99.13%	2553	HQ711852.1
Streptococcus mutans dexA gene for dextranase, complete cds	100%	0.0	99.13%	3747	D49430.1

Figure (3): The Blast Report of sequences producing significant alignment of S.mutans isolate sequence with 16 reference sequence of S.mutans deposited in genbank with accretion numbers, Identity about 99 % for each.

Genotyping S. mutans isolates based on Sequence analysis.

Nine strains of S.mutans were subjected for sequence analysis; the results of sequence were subjected for multiple alignments by Geneious prime software. Four variants were observed with high frequency, variant 1 wild type T mutant to G (T>G) with frequency 33.4%; variant 2 C>t (44.4%); variant 3 A>C (66.6%) and Variant 4 C>T (44.4%). The Frequency values included homozygous mutation and heterozygous mutation. Figure 4.

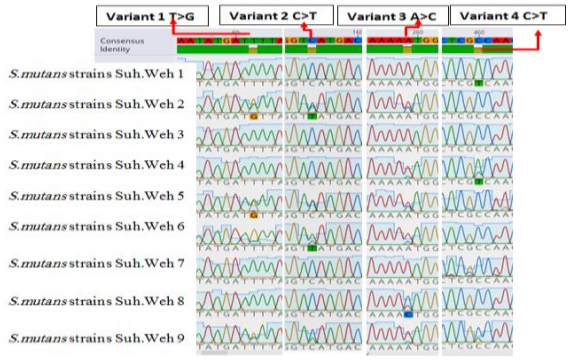

Figure 4: The multiple alignments of chromatograms of targeted region 1169-1773 had shown four genotypes. The alignment performed by Geneious prime software.

The results of multiple alignments based on Geneious prime software shown highly sharp view of micro-evolutionary in 9 strain of S.mutans, in genotype one the 3 out of nine shown heterozygous mutations from thymine to guanine, genotype 2 shown 3 strains undergo mutation cytosine to thymine (C>T), genotype 3 shown 6 mutants out of nine strains, the homozygous and heterozygous mutant allele from adenine to cytosine (A>C), while the genotype 4 shown mutation in four strain, homozygous in strain one while the other were heterozygous mutation from cytosine to thymine (C>T) (Figure 4).

Phylogeny tree of S. mutans based on multiple alignment sequence analysis online and based on MEGA 6 software:

The eight and nine sequences of S. mutans isolates were constructed phylogeny tree by MEGA 6 software (Figure 5), with close related bacterial sequences to construct the phylogeny tree to provided two aims, First to confirmed the specify of primer pair designed for S.mutans nor other Streptococcus spp. or any other bacterial spp.. second, the result shown the S. mutans stain 1 and 9 came with reference strains of S.mutans with accession numbers CP01323.1,CP033199.1,LR13432.1,CP033744.1, and AP014612.1in the closed cluster , and conformed high identity more than 99% among nine strain, while others S.pneumoniae became as different tree root with distance about 0.71 , Figure(5).

The result of construction phylogeny tree 9 S.mutans FedxA gene sequence based on Mega 6 software, shown all strains came closed to each other. This result explains that all strains as one biological unit compared with tree root representative by S. pneumoniae as different species Figure (5).

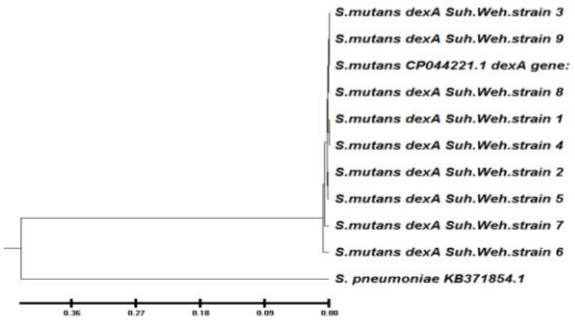

Figure 5: Construction of phylogeny tree by Mega 6 UPGM of nine S.mutans sequences of dexA gene alignment with reference strain S.mutans CP044221.1 and tree root with S.pneumoniae KB371854.1.

This study recognized many strains of S. mutans patients caused dental plaque (matured 20-60 years) in Iraq. As per the other epidemiological information in Iraq, S. mutans were distinguished in 93.1 % of the dental plaque tests, individually, based on phenotypic and molecular tests, our results agree with results of Palmer et al., (2013). The recognition fre-quency of the two living beings was accordingly higher than in our information. This may be because of the various times of the subjects utilized. In a new report, the predominance of S. mutans was accounted for to be 72.8% in 77 Japanese matured 3-5 years of age, by utilizing a PCR technique (Okada et al., 2002). Meng et al.,2013 additionally revealed the predominance of S. mutans in dental plaque tests to be 75.4%, utilizing biochemical tests and PCR. Likewise, S. mutans was recognized in 65-95% of pre-younger students matured 5-13 years of age of the United States and Canada, individually.

During this study we recognition strategies utilized were unique, the dispersion and prevalence of S. mutans detailed in the investigations referred to above, including our own show a comparable propensity to those of the current review.

The results of this study shown high polymorphism in S. mutans based on sequence methods, this genetic approach was considered as golden method for tracking polymorphism compared with RFLP-PCR method or RAPD-PCR method. Many previous

studies followed low productivity genetic methods like RFLP-PCR. The dex-designated PCR-RFLP and biochemical tests. Another studies used universal primer pair like s16 RNA the screened strains were distinguished at the species level utilizing the 16S rDNA sequencing correlation strategy. Strangely, the after effect of 16S rDNA sequencing examination investigation was plainly predictable with that of PCR-RFLP examination. Consequently, dex-designated PCR-RFLP could be utilized to recognize the mutans streptococci without the requirement for 16S rDNA sequencing examination investigation. The clinical secludes that were not recognized as mutans streptococci by PCR-RFLP were grouped into non-mutans streptococcal creatures (Yoo et al., 2005).

The direct genotyping based on PCR banding methods for clinical models of caries nebulae is a pioneering and rapid method that bypasses the multi-step implantation, isolation, extraction, and sampling of the microbe of interest. the follow direct genotyping method that is adjusted to a wide assortment of test types and avoid DNA extraction is testing since current systems for genotyping dependent on PCR require moderately high pure of DNA. Be that as it may, tests normally utilized in clinical settings, for example salivation, dried blood spot , and buccal swab, are difficult to genotype without DNA extract since cell layers forestall cell harm and DNA extraction. Consequently, fostering an overall methodology for a genotyping method that dissects different example types without DNA extraction has stayed subtle. Albeit a few works that perform PCR enhancement without DNA extraction from salivation (Ambers et al., 2018) have been created, they actually require extra expert, considerable power utilization.

We applied one further developed advance into one framework: a direct PCR examine for DNA enhancement without culturing methods extraction framework to peruse the outcomes quickly results and reduced costs. These commitments address a few bottlenecks of current strategies while giving the benefits of effortlessness, cost, movability, and quantitative genotyping. This innovation is one bit nearer to understanding the omnipresent accessibility of quality tests, which can at last guide fast clinical choices.

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