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Diagnosis and detection of VicK gene in *Streptococcus mutans* isolated from the saliva of patients with diabetic type 2 with tooth decay in the Iraqi population

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ABSTRACT

Type 2 diabetes mellitus (T2DM) is gradually becoming more common in Iraq. Salivary changes and proliferation of specific bacterial communities cause oral disease that can adversely affect systemic conditions such as diabetes. Fifty saliva samples were collected from people with T2DM suffering from tooth decay and twenty-five people without T2DM suffering from tooth decay. The periodontal status, the extent of the root surface, and coronal caries were evaluated. Saliva was cultured for investigating Streptococcus mutans. The results showed that patients with type 2 diabetes had significantly more severe Periodontitis and a higher prevalence and magnitude of bacterial caries. Diabetic subjects had higher levels of Hemoglobin A1c (HbA1c) and Random Blood Sugar (R.B.S.). The *S. mutans* diagnosis by PCR for Sanger Sequencing technique by using VicK gene sequences (1300bp). The PCR products of the isolate were submitted to Macrogen Company for sequencing. Selected seven isolates as new isolates registered in global gene bank as locally *S. Mutans* isolates in Bagdad city/Iraq and their accepted accession numbers include LOCUS MT603520, MT603521, MT603522, MT603523, MT603524, MT603525,and MT603526 of nucleotide sequence. The VicK genes isolates' phylogenetic trees revealed a genotype that was closely connected to other isolates in GenBank. Furthermore, gene sequencing demonstrated a success rate of 99 percent. resemblance to other isolates in the GenBank database The likelihood of a link between S. Mutans and dental carries was determined by these findings.

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Introduction

Diabetes mellitus (D.M.) is a group of metabolic diseases with hyperglycemia caused by insulin action and/or insulin secretion defects (1). Type 1 diabetes, type 2 diabetes, and gestational diabetes are the three main types of diabetes (2). Type 2 diabetes is the most common. Chronic hyperglycemia is linked with long-term tissue damage, which results in dysfunction and failure of different organs (3,4) and an increased risk of oral diseases, inclusively periodontal disease (5,6), dental caries (7), and xerostomia (8). Diabetes mellitus causes structural and functional changes in susceptible tissues, leading to certain complications in type 1 D.M. and type 2 D.M. Individuals with no functional metabolic abnormalities, insulin deficiency, and hyperglycemia are generally at low risk for developing these complications. Oral health depends on the stability of microbial communities, and oral disease occurs when pathogenic species outnumber the normal flora (9). Pathogenic microorganisms in the oral cavity are frequently linked to two primary diseases: dental caries and periodontal disease (10).

Non-enzymatic glycosylation (11), altered hemodynamics (12), and genetic factors are involved in the development of D.M. (13–16). However, it is not clear which factors or combinations of mechanisms are directly responsible for complications in the target tissues. It is also not clear whether a different type of mechanism is operating in different tissues. However, it is known from prior studies that not all people with diabetes mellitus experience these complications; also, there is a variation in the developmental rate and severity of these complications.

Many studies have supported the idea that patients with diabetes mellitus are at higher risk of developing periodontitis. In other words, periodontal disorder is

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considered an additional complication of diabetes (17). Periodontal disease is a widespread chronic inflammatory disorder in which the supporting structures of teeth are damaged (the periodontal ligament and alveolar bone). It is widespread (severe periodontitis affects 10-15% of adults) and has many detrimental effects on life quality. The relationship between the degree of hyperglycemia and the severity of Periodontitis is evident. The underlying mechanisms of the association between these two conditions are not entirely known but include aspects of immune functioning, cytokine biology, and neutrophil development. Evidence is emerging to support the presence of a two-way relationship between diabetes and periodontitis, as diabetes enhances the risk of periodontitis and, on the other hand, glycemic regulation is adversely affected by periodontal inflammation. The prevalence of macroalbuminuria and end-stage renal disease is two to three times higher in diabetics with severe periodontitis than in diabetics without severe periodontitis. Various researches had conducted to find out the relationship between glycemic control and Periodontitis. Findings suggest that poor glycemic control is associated with an increased risk of periodontitis (18–25). However, multiple studies have reported no association between glycemic control and periodontitis (17,26–32). Most of these researches usually investigated type 1 diabetes mellitus or do not determine the type of DM. Many of them failed to estimate the glycemic control-related risk of periodontal disorder as they are cross-sectional studies. Limited studies used multivariate analysis, but no study has used teeth or oral health as analysis components for associated observation in their statistical analysis. In spite of very little research attention, researchers investigated the possible relation of dental caries with diabetes (33,34). The outcomes confirmed that there is no difference between people with diabetes and healthy individuals in Finland in the incidence of dental caries, while another study indicated that diabetes was a significant risk agent for root caries (7). Although the causes for the increased incidence of diabetic dental caries remain unclear (34), many oral pathogens, like *Streptococcus mutans* (*S. mutans*) and lactobacilli in the saliva, may be associated with this. However, in two separate studies, reports on *S. mutans* and lactobacilli in the saliva of diabetic and non-diabetic individuals were not substantially different (7). Vic-genes regulate the expression of multiple genes associated with virulence (35). Furthermore, VicK inactivation results in a decreased level of lactic acid and an increase in acid tolerance for S. Mutans (36). A VicK knockout mutant was found to be more responsive to H2O2 than the wild-type mutant (37).

The aim of this study was the evaluation the effects of Diabetic Mellitus on dental caries and specifically Streptococcus mutans and investigation of their phylogenetic tree.

Materials and methods

Ethical consideration

The bacterial strains used in this research were extracted from clinical routine specimens, and patients were given verbal consent. This study has been accepted by the College of Health Sciences / Hawler Medical University Scientific and Research Ethics Committee

Sample Collection

A total of fifty patients with diabetes mellitus and tooth decay had classified into two groups (group1: patients with T2DM for ten years, and group 2: patients with T2DM for 15 years), and also, as a control group, twenty-five individuals were attended in this study. They were examined in National Diabetes Center, Mustansirya University, Baghdad, Iraq, between September 2019 and January 2020. The Hemoglobin A1c (HbA1c) and Random Blood Sugar (R.B.S.) were evaluated by BioSystems Analyzer (A15 Automated Analyzer). Careful history was obtained from both groups according to a questionnaire that covers all information, including name, age, family history, sex, smoking, drinking alcohol and any other diseases after diagnosis by medical staff.

Culture of sample and Morphology of bacteria

Saliva samples were taken from carious lesions of fasting patients who had their usual examination in the morning using the ends of sterile wooden toothpicks. Before collecting the samples, they were told not to brush their teeth for at least an hour. To avoid dilution of samples, the individuals were instructed to rinse their mouth with distilled water and wait at least 5

minutes. The toothpicks were cut off and dipped in 1 mL of sterile phosphate-buffered saline (PBS) (HiMedia, India) before being stored at 4 °C. To scatter the plaque and achieve a homogenous suspension, saliva samples were vortexed for one minute. The samples were diluted 100-fold in 1x sterile PBS and plated on Mitis Salivarius Bacitracin (M.S.B.) agar. Salivarius agar mitis (HiMedia, India) was used to make the M.S.B. agar, which was supplemented with 15% sucrose, 1% agar, 0.0001% potassium tellurite solution, and 0.2 units/ml bacitracin (HiMedia, India). The plates were incubated anaerobically at 37°C for 48 h. The colonies were recognized based on colony morphology after the incubation phase. Each sample plate's typical colonies were transferred to brain–heart infusion (B.H.I.) broth (HiMedia, India) and cultured for 18 hours at 37°C. The broth cultures were streaked on M.S.B. agar and anaerobically incubated at 37°C for 48 hours after the incubation period. The overnight bacterial cultures were kept at 20°C in a stock of 80 percent glycerol (38).

16S rRNA PCR for *Streptococcus mutans* identification

The identity of Streptococcus mutans was verified via po¬lymerase chain reaction (PCR). All primers for the detection of 16SrRNA genes were designed using the NCBI primer designing tool. The primers were synthesized and provided by Bioneer Company, Korea (F: GTTTACGGCGTGGACTACCA and R: CCACACTGGGACTGAGACAC). The final volume of the PCR reaction was 25 µL using 12.5 µL of 2x Hot Start Taq Master Mix, 1 µL of the DNA template, 1 µL of each primer (20 pmol) and 9.5 µL of ddH2O. DNA amplification was done in a thermocycler PCR. Amplified products were subjected to electrophoresis using 1.2% gel agarose (GeNet Bio, Korea) (Figure 1).

Molecular diagnosis of VicK gene using PCR technique

Total DNA of Bacteria isolates were extracted using DNA Extraction Mini Kit (17045/ Intron biotechnology/Korea). The VicK gene was amplified using the primer F (5'-CGGGATCCATGACTAATGTGTTTGAATCAAGTC -3') and R (5'-CCGCTCGAGTCATGATTCGTCTTCATCTTCTTCC -3') (36). The PCR amplification was done in a total volume of 25µl containing 1.5µl DNA, 5µl Taq PCR Pre Mix, 1µl of each 16.5 pmol primers which is applied in a tube with a total volume of 25µl then nuclease-free. Thermo cycling conditions were as seen in Table 1.

Table 1. PCR program that was applied in the thermocycler devices

Steps	Temperature	Time	No. of Cycles
Denaturation 1	95˚C	5min	1
Denaturation 2	95˚C	45sec	35
Annealing	48˚C	45sec	
Extension 1	72˚C	45sec	
Extension 2	72˚C	7min	1

Sequence of gene

VicK gene sequencing was conducted at Macrogen Company using the ABI 3730xl genetic analyzer (Applied Biosystems, U.S.A). Homology searches were carried out online at the National Center for Biotechnology Information (NCBI) at (http:/www.ncbi.nlm.nih.gov) and Bio Edit utilizing the Basic Local Alignment Search Tool (BLAST) program. The outcomes were compared with information accessible online at the NCBI from the ExPASY program released by Gene Bank.

Results and discussion

This study detected VicK gene in samples collected from the saliva of patients with T2DM. PCR products showed that 3 (30%) out of 10 positive samples of S. Mutans in G1 group were positive for VicK gene and 5 (38.4%) out of 13 isolates in group 2 were positive for this gene as seen in Table 2.

Table 2. Distribution of positive *Streptococcus mutans* isolates.

Isolates	No. of isolate (%)	No. of Morphology of *S. Mutans* (%)	No. of positive VicK gene product (%)
G1 group	25 (33.3%)	10 (40%)	3 (30%)
G2 group	25 (33.3%)	13 (52%)	5 (38.4%)
Control group	25 (33.3%)	3 (12%)	0

All isolates in our study have been investigated for detection of *S. mutans* by 16S rRNA genes (Figure 1) with a product size of 120 bp. 16S rRNA-based PCR assays quickly, easily and reliably identify *Streptococcus mutans* and distinguish them from other phylogenetically related Pseudomonas spp. Assays have a sensitivity and accuracy of 100 % of intended targets. Amplification of 16S rRNA from 22 isolates was performed to confirm bacterial identification. Primers for the conserved region of 16S rRNA were designed and used for PCR amplification of DNA of *S. mutans* isolates. Then PCR products were separated on agarose gel (Figure 1). The result demonstrated that 10(33%) of *S. mutans* had 16S rRNA gene band with 120 bp. The result shows that patients with T2DM have significantly higher Hemoglobin A1c (mmol/ml) levels compared to the control group (Figure 2). The results, however, revealed no significant differences between T2DM groups (group1: patients with type 2 diabetes for up to 10 years, and group2: patients with type 2 diabetes for 15 years or more).

The values of serum Random Blood Sugar (mg/dl), which are illustrated in Figure 3, revealed a significantly higher level in the patient's groups compared to the control group. The polymerase chain reaction diagnostic techniques are rapid, easy, inexpensive protocols, becoming the most commonly utilized molecular genetics method for detecting essential genes and identifying the bacteria. The results of PCR amplification of VicK gene (1300bp) were performed and results are shown in Figure 4.

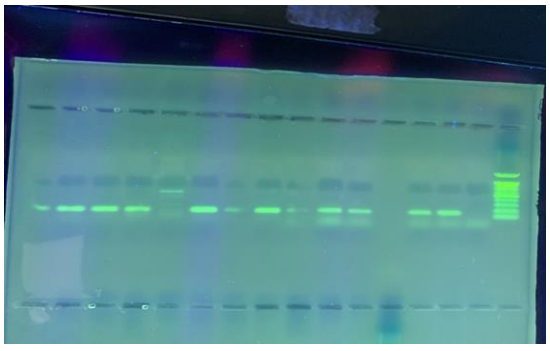

Figure 1. Agarose gel electrophoresis of PCR products after amplification of the 16S rRNA gene (120 bp). Lane M: 100 bp DNA ladder. Lane 1: Negative control. Lane 4, 7 and 11: Negative control. Lanes 2,3,5,,6,,8,9,10,13,14,15: Positives for *Streptococcus mutans*

The phylogenetic tree in Figure 5 was generated with the Molecular Evolutionary Genetics Analysis (MEGA) software version 6.0. For phylogenetic analysis, a neighbor-joining tree was built (Figure 5).

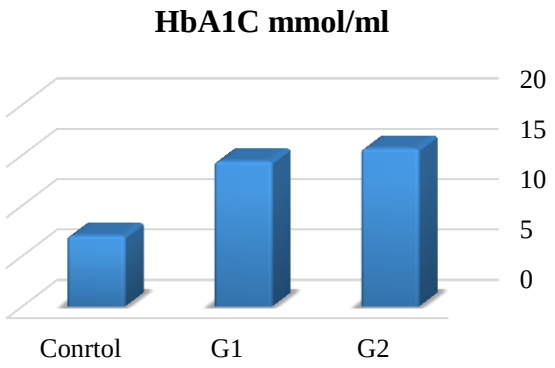

Figure 2. Levels of HbA1C (mmol/ml) of T2DM patients and control subjects

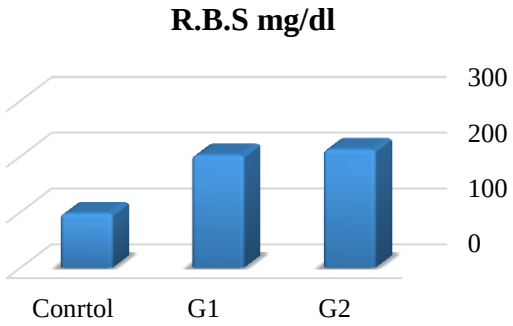

Figure 3. Levels of serum R.B.S (mg/dl) of T2DM patients and control healthy subjects

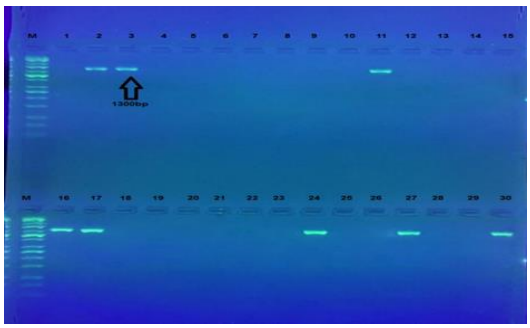

Figure 4. Agarose gel electrophoresis for VicK *gene* (1300bp). Bands were fractionated by electrophoresis on 2% agarose gel (2 h., 5V/cm, 1X T.B.E.) and visualized under U.V. light after staining with a red stain. Lane M: 100bp ladder, Lane 2, 3 and 11: Positive PCR product of G1, Lane 16, 17, 24, 27 and 30: Positive PCR product of G2

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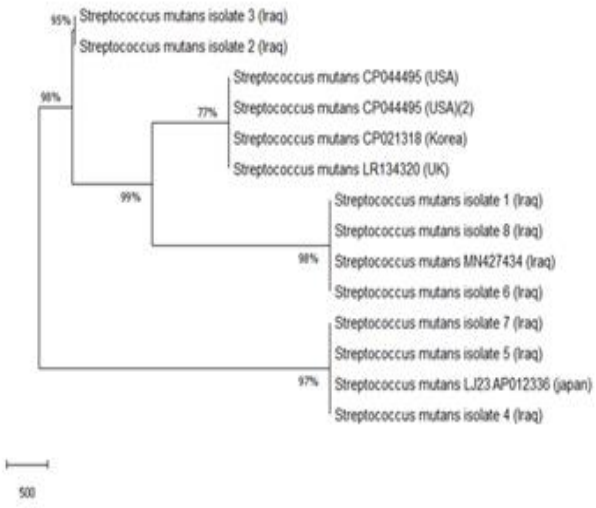

Figure 5. Neighbor-joining tree *Streptococcus mutans* of VicK gene

The results of alignments demonstrated that the *Streptococcus mutans* of Iraq and other global strains show partial sequence similarity in translating specific regions of the VicK gene. Hierarchical cluster analysis determines the clusters including *Streptococcus mutans* Iraq isolates of 2 and 3 with the identical percentage of 95%, isolates of 1,4,5,6,7 and 8 with the identical percentage of 97%, and 98 % it is close to Iraq (ID: MN427434) the with the identical percent of 98%.

Distance matrices and the results of BLAST which compared the *VicK* gene isolated from the *Streptococcus mutans* in this study other global isolates are presented in Figure 6.

The sequencing of the VicK gene was performed by the Macrogen Company. The findings were compared with data obtained from the ExPASY system published by Gene Bank available online at the NCBI. The results of *S. Mutans* positive isolates showed that more variation in isolates was seen in group2 which showed 11 variations including five Transversion and six Transition. On the other hand, group 1 showed eight variations including two Transversion and six Transition which 98% identified with the standard of Gene Bank as was shown in Table (3).

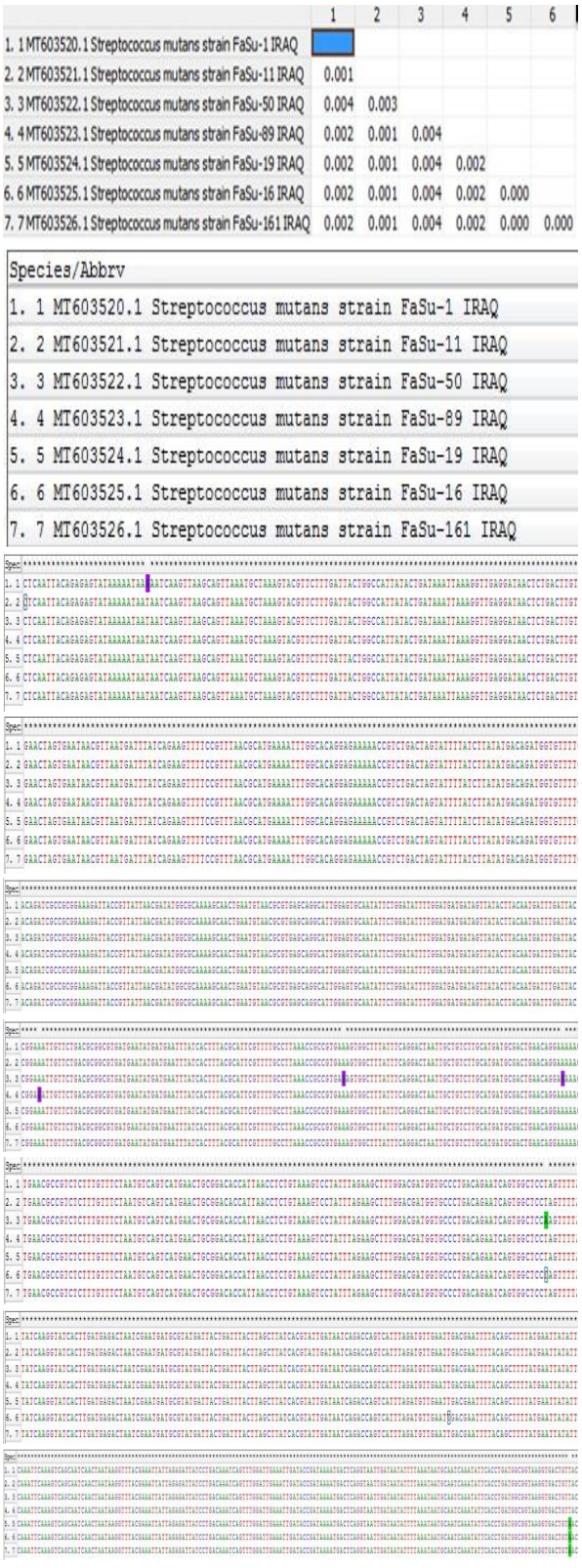

Figure 6. Distance matrices and the results of BLAST which compared the *VicK* gene isolated from the *Streptococcus mutans* in this study to other global isolates

Table 3. Represents the type of polymorphism from VicK gene from *Streptococcus mutans* for isolates Group 1 and Group 2.

No. of sample	Type of substitution	Location	Nucleotide	Sequence ID of gene Bank	Sequence ID of Iraqi Strain
Group1	Transition	136	A>G	CP050273.1	MT461698.1
	Transition	704	A>G		
	Transition	877	A>G		
	Transition	993	A>G		
	Transversion	997	C>G		
	Transition	1001	C>T		
	Transversion	1012	T>A		
	Transition	1029	T>C		
Group 2	Transition	136	A>G	AP012336	
	Transversion	241	A>T		
	Transversion	244	A>T		
	Transversion	264	T>A		
	Transition	704	A>G		
	Transition	877	A>G		
	Transition	993	A>G		
	Transversion	997	C>G		
	Transition	1001	C>T		
	Transversion	1012	T>A		
	Transition	1029	T>C		

The current investigation offers information on the connection between diabetes and oral disease. There is a link between bacterial numbers and active caries in individuals with diabetes. The higher frequency of functional dentistry caries in patients with diabetes than non-diabetics patients was supported by previous studies (7).

Diabetes mellitus is a disorder that causes elevated glucose levels in the blood resulting from decreased blood insulin levels. This causes several metabolic defects in the carbohydrate, fat, and protein pathways (39). HbA1c assays measure the amount of haemoglobin glycated in the blood and provide a reliable estimation of blood glucose regulation over the last 1 to 3 months. Glycation gradually appears in about 2 to 3 months and recurs early in its development and remains constant until the regeneration of red blood cells (R.B.C). That's why HbA1c has been used to monitor people with diabetes as an index of long-term glycemic control (40). D.M. also affects the mouth and can lead to dental caries, periodontitis, low salivary level, oral mucosal diseases, and infections such as lichen planus, recurrent aphthous stomatitis and candidiasis. Oral health is vital for a person with diabetes. Epidemiological studies indicate that diabetes is a major risk factor for periodontitis and that if glycaemic regulation is low, the risk of periodontitis is higher. People with poorly controlled diabetes (who are also at great risk of other macrovascular and microvascular complications) are at increased risk of periodontitis and loss of alveolar bone (41,42). Given the expected rises in the prevalence of diabetes over the next few decades, the previously observed declines in the majority of Periodontitis (associated with less smoking and improved oral health behavior in recent years) are likely to be reversed as a consequence of a significant rise in the number of people with diabetes (43). It is possible that diabetes

management (i.e., improving glycaemic control) would decrease the risk and severity of Periodontitis. Evidence indicates that periodontal inflammation resolution may improve metabolic control (with approximately 0.4 percent reduction of HbA1c reported), although massive, multi-center, randomized controlled trials are required to validate these findings further.

The main symptoms of periodontal disease are gingival bleeding, and the saliva's decreased flow rate is a vital risk agent for oral candidiasis (44). Dental carries and D.M. have a complicated relationship. Diets that restrict carbohydrate intake are commonly prescribed for children with T1DM., cariogenic foods, T2DM is primarily connected with obesity and a high-calorie, carbohydrate-rich diet in children and adults. There is no clear trend in the literature concerning the connection between dental caries and diabetes (45).

A recent study in Saudi Arabia has shown a high prevalence of oral bacterial in subgingival pockets of T2DM patients as compared to normal subjects (46). The *streptococcus mutans* were selected based on previous studies. For example, a study that tested different patients with dental caries accompanied by diabetes mellitus showed significantly high numbers of streptococcus mutans compared to other bacterial infections (47). Furthermore, another study demonstrated that patients who suffered from dental plaque and were positive for *S. Mutans* showed higher caries incidence (48). S. Mutans from dental carries in Iraq was investigated previously by many studies and had been shown various frequencies. A study was done in the Thi-Qar governorate in Iraq and the data demonstrated 33 (41%) S.mutans isolates were isolated from 80 dental plaque individuals aged between 7 to 15 years (49). Mahdi (2015) in Kufa, Iraq, reported that the ratio of S.mutans in dental caries patients is 40%. In a study conducted in Saudi Arabia, Almusawi et al.(2020) discovered that a considerable number of T2DM patients(78%) had high counts of streptococcus mutans in their saliva (105 CFU/ml) and significant relationships between streptococcus mutans load and diabetes., saliva flow rate, saliva buffering capacity, and glycemic control (50). Streptococcus mutans thrived in the saliva of T2DM patients because of hyposalivation, increased salivary glucose, and poor glycemic control. In this study, we obtained a ratio of 40% of S.mutans isolates in the first group and 52% in the second group. The PCR amplification has confirmed the results and showed 33.3% in both groups, which is lower than previously reported in studies.

Identification of *S. mutans* isolates using 16S rRNA is more accurate than bacteriological and biochemical assays. Rampini *et al* (2011) demonstrate that 16S rRNA gene PCR which was sensitive and specific to use for diagnosis of culture-negative bacterial infections is also useful for identification of bacterial pathogens in patients pretreated with antibiotics (51).

One *S. mutans* bacterial isolate from Iraq has been registered in the global gene bank after examination of the VicK gene sequence. Locally *S. mutans* isolates in Baghdad city which is not similar to global isolates can be considered as new isolates. Therefore, selected isolates were registered as new isolates in the global gene bank and their accepted accession numbers include LOCUS MT603520, MT603521, MT603522, MT603523, MT603524, MT603525, and MT603526 of the nucleotide sequence. The phylogenetic trees for the VicK gene, which encodes the *S. mutans*, revealed a genotype that was closely connected to others in Gen Bank. Gene sequencing found 99 percent similarity with other isolates in GenBank, similar to the *S.mutans* gene.

In this study, we sequenced the vicK gene of *S. Mutans* strains isolated from two groups of patients with dental caries to analyze the effects of vicK polymorphisms. VicK gene is responsible for regulating the expression of multiple virulence-associated genes, which affect polysaccharide synthesis and adhesion. Additionally, vicK inactivation produces a reduced level of lactic acid. It improves acid tolerance *S. Mutans,* so any polymorphism account in this gene can affect the surveillance and virulence of *S. Mutans.* The capacity of *S.Mutans* to consume glucose as a result of carbohydrate metabolism to generate lactate is a crucial virulence factor associated with this pathogen. It was shown that the inactive Vick gene could reduce acid production, which affects bacterial surveillance.

In this study, both groups' sequencing results showed variations in the vicK gene. The first group showed 11 variations, while the second group showed eight variations. These results indicated the probability of the relationship between *S. Mutans* and dental carriers. Clinical isolates of mutans and the

vicK genes C470 T missense mutation may be linked to the caries experience with S. mutans. The phylogenetic analysis was done for geographic genetic distance determination. The analyzes of phylogenic distance occurred for partial-length of vicK. The results provided a clear picture of genetic distance. The isolates 2 and 3 showed the same distance, and they differ from the other Iraqi isolate published previously (I.D.; MN427434) by only 3%. We can suggest from the results of this study that variation is dependent on how antibiotic treatment can be effective.

Conclusion

These results indicated High prevalence of oral bacterial *S. Mutans* in Diabetic patients and the probability of the relationship between *S. Mutans* and dental carries and clinical isolates of mutans and the vicK genes missense mutation may be linked to the caries experience with *S. mutans*.

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None

Interest conflict

The authors declare no conflict of interest.

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