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DETECTION OF *gtfs* GENES WITH DENTAL CARIES IN SOME IRAQI DIABETIC PATIENTS

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ABSTRACT
A study was conducted to investigate the validation of the molecular PCR technique as a diagnostic method to identify the causative-agents bacteria which was associated with oral streptococci present in the mouth using species specific primer *gtfs* and sequencing. The total number of the studied groups is 95 Iraqi patients (45 diabetic patients DP and 50 non-diabetic patients NDP) of both genders. Ages ranged from 18-65 years old. PCR results revealed that the presence of the product with 433, 544, and 374 bp in size which was related to *gtfD* (*S. mutans*), *gtfK* (*S. salivarius*) and *gtfR* (*S. oralis*) genes respectively in all samples (saliva and swabs). The sequencing analysis of *gtf*s gene (*gtfD*, *gtfK* and *gtfR*) showed that the highest percentage of recorded mutations was in the *gtfR* gene in DP, while that was in the *gtfK* gene in NDP. All mutations in *gtfR* gene were of substitution type in DP and NDP. In DP, the mutations in *gtfK* and *gtfD* genes were distributed in to substitution and insertion mutations without recording any type of deletion mutation. But, in NDP mutations were distributed between three types of mutations (substitution, insertion and deletion).The silent and missense mutations in the three genes *gtfD, gtfR* and *gtfK* for DP patients had the same percentage and it was higher than the frameshift mutations. Whereas, the highest percentage was recorded missense mutations in NDP as compared with the two other types mutations (silent and frameshift).

KEY WORD: Diabetes mellitus, Dental caries, Oral Streptococci, PCR technique, Sequencing.

INTRODUCTION

Most oral streptococci possess glucosyltransferase (GTF) enzyme that use sucrose as a substrate to synthesize extracellular polysaccharide (glucans), which is an obligate factor for biofilm formation of dental plaque, and which facilitates the adhesion and accumulation of oral bacterial cells to tooth surfaces (Maria *et al*., 2010). Diabetic mellitus is one of the serious chronic systemic disorders of glucose metabolism that may cause general systemic changes, which may be reflected in the oral cavity (Guyton and Hall, 2012). Dental caries is a multi-factorial disease, it has been found to be more common and more severe in diabetic patients than in non-diabetic patients (Jaweda *et al*., 2011). Dental caries are among the most common diseases worldwide, and are caused by a mixture of microorganisms, colonized in the dental surface and cause damage to the hard tooth structure in the presence of fermentable carbohydrates *e.g.*, sucrose and fructose to organic acids. These acids decalcify the tooth enamel and lead to destruction of tooth hard tissue and consequently tooth decay (Sofia *et al.*, 2010). The oral cavity contains the greatest biodiversity, over 70 species being isolated from mouth mucosa, saliva, denture surfaces and dental-plaque (Damian *et al*., 2010). Microbiological strategies to identify associations related to dental caries are limited due to the lack of highly selective media formulations and the un-cultivability of many oral species (Kanasi *et al*., 2010). Since both commensal and cariogenic streptococci have GTF enzymes, the proposed detection would help determine bacterial composition of the oral flora (Hoshino *et al*., 2012). PCR method is useful for the analysis of oral streptococci and can be successfully used in clinical applications to identify pathogenic bacteria associated with oral infectious disease. PCR technique using species-specific primers (*gtfD, gtfK, gtfR*) which target almost the full length of the analyzed *gtfs* gene were applied to identify different bacterial cells species, which are *S. mutans, S. salivarius*, and *S. oralis* respectively (Hoshino *et al.*, 2004; Eriko *et al.*, 2009). This study aimed to differentiation of different type of oral Streptococci which present in the mouth using *gtfs* gene by PCR technique. Studying the relationship between diabetes disease and dental caries in comparison to non-diabetic patients and using PCR technique as diagnosis method to identify the causative-agents bacteria which are associated with oral disease using species specific primer and sequencing.

MATERIALS & METHODS

A total of the studied groups consist of 95 Iraqi patients (45 diabetic patients and 50 non-diabetic patients) for both genders aged from 18-65 years old. The samples (saliva (1ml) and swabs from oral cavity) were randomly collected from DP and NDP patients who are reviewing Al-Alweyia Center of Dental Caries and Diabetic Diseases in Al-Yarmook hospital in Baghdad city. Nb. swabs were put in test tubes containing 5ml of Phosphate buffer slain (PBS).

Genotyping

All subjects' samples (saliva and swab), which were collected from oral cavity were transported to the laboratory immediately for culturing and extracting the total bacterial genomic DNA using genomic DNA extraction Mini kit (BioNeer, Korea). PCR technique was

used to detect the *gtf*s genes using species-specific primers targeting almost the full length of the analyzed gene (Table1). PCR Programs: Each forward and reverse primer (0.7µl) were put in an eppendorf tubes, then Template DNA (5µl), and 12.5 master mix (Promega, U.S.A) were added, and the volume was completed to 25µl by adding 6.1µl free nuclease distilled water. The PCR amplification reaction was performed in a thermal cycler (Promega, U.S.A) with the following cycling parameters: an initial DNA denaturation at 95^{o}C for 5 min was carried out for 1 cycle and then 38 PCR cycles consisting of denaturation at 94^{o}C for 22 sec., annealing for 1 mint. at (69, 68, 66^{o}C) for (*gtfD, gtfK, gtfR*) respectively and extension at 72 ^{o}C for 37 sec. and final extension at 72 ^{o}C were carried out for 2 min. The PCR products were subjected to electrophoresis in a 2% agarose gel-TBE buffer. The gel was stained with 0.5 µg of ethidium bromide per ml and photographed under UV illumination. A 100-bp DNA ladder (promiga, USA) was used as the molecular size standard.

TABLE 1: Primers for Detection of *gtf* Genes in Streptococci spp. used in this study

PCR Product size	Sequence of primers (Tomonori *et al.*, 2004; Eriko *et al.*, 2009) BioNeer (Korea)5'-3'	Primer	
	GGCACCACAACATTGGGAAGCTCAGTT	Forward	*S. mutants*
433	GGAATGGCCGTAAGCTAACAGGAT	Reveres	*gtfD*
	GTGTTGCCACATCTTCACTCGCTTCGG	Forward	*S. salivarius*
544	CGTTGATGTGCTTGAAAGGGCACCATT	Reveres	*gtfK*
	TCCCGGTCAGCAAACTCCAGCC	Forward	*S. oralis*
374	GCAACCTTTGGATTTGCAAC	Reveres	*gtfR*

Sequencing PCR products

The PCR products of 12 samples for the *gtf*s gene and primers were sent to Macrogen Company (U.S.A) by Alexander Company (sadoun street–Baghdad- Iraq) for sequencing as fellow (4 samples of each primer; 2 samples from diabetic patients and 2 samples from non-diabetic patients). Each forward and reverse primer (0.7µl) were put in an eppendorf tubes containing lyophilized master mix (BioNeer, Korea) then Template DNA (5µl) was added. The volume was completed to 20µl by adding 13.6µl free nuclease distilled water.

Statistical analysis

Data were analyzed by using SAS program (2010). Chi-square test was used to compare the differences between proportions.

RESULTS & DISCUSSION

PCR analysis

PCR amplified regions of *gtfD, gtfK*, and *gtfR* in *S. mutans*, *S. salivarius* and *S. oralis* are showed as bands with a molecular weight of 433bp, 544 bp and 374 bp, respectively as compared with 100bp DNA ladder from the samples (saliva and swabs) for diabetic and non-diabetic patients. Fig. 1, 2, 3.

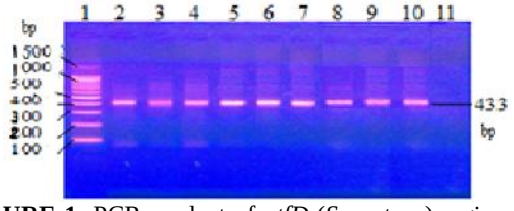

FIGURE 1: PCR product of *gtfD* (*S. mutans*) region 433 bp electrophoresed on 2% agarose gel 5volt/cm^2. Lane 1: ladder Marker (100bp), Lane 2,3,4,5, and 6: from DP samples; Lane: 7, 8, 9 and 10 from NDP samples and Lane 11: negative control * DP: Diabetic patients NDP: Non-diabetic patients.

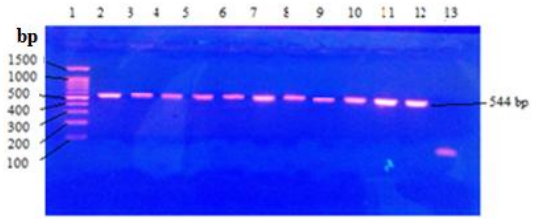

FIGURE 2: PCR product of *gtfK* (*S. salivarius*) region 544 bp electrophoresed on 2% agarose gel 5volt/cm^2. Lane 1: ladder Marker (100bp), Lane 2,3,4,5, and 6: PCR product from DP samples; Lane:7,8,9,10,11 and 12: PCR product from NDP samples and Lane 13: negative control. * DP: Diabetic patients NDP: Non-diabetic patients

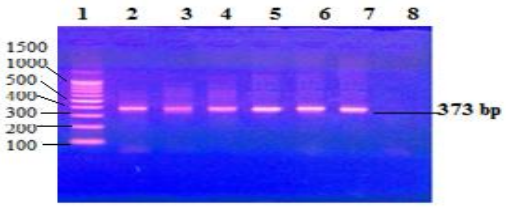

FIGURE3: PCR product of *gtfR* (*S. oralis*) region 374 bp electrophoresed on 2% agarose gel 5volt/cm^2. Lane 1: ladder Marker (100bp), Lane 2, 3 and 4: from DP samples 5,6 and 7: from NDP samples and Lane 8: negative control * DP: Diabetic patients NDP: Non-diabetic patients

Depending on the results detection the *gtfs* gene, *gtfD* (*S. mutans*), *gtfK* (*S. salivarius*) and *gtfR* (*S. oralis*)) in all samples (saliva and swabs) (Table 2 and 3).There were high significant differences at ($p<0.01$) between diabetic patients than non-diabetic patients, while, there were no significant differences among the results of the three species of bacteria *i.e.*, the present study, document major cause of dental caries are *S. mutans*, *S. salivarius* and *S. oralis* in diabetic patients . Dental caries and dental plaque are among the most common diseases worldwide, and are caused by a mixture of microorganisms and many factors.

TABLE 2: Distribution of *gtfD*, *gtfK* and *gtfR* in diabetic and non-diabetic patients groups according to saliva samples

Chi-square value	Saliva in NDP#		Saliva in DP^		Bacteria (primer)
	%	No./50	%	No./45	
7.944 **	60	30	91.11	41	*S. mutans* (*gtfD*)
8.364 **	54	27	84.44	38	*S. salivarius (gtfK)*
8.509 **	58	29	86.66	39	*S. oralis (gtfR)*
---	1.314 NS	---	1.278 NS	---	Chi-square value

**: high significant difference (P<0.01 NS: no significant ^: Diabetic Patient #: Non- Diabetic Patient

TABLE 3: Distribution of *gtfD*, *gtfK* and *gtfR* in diabetic and non-diabetic patients groups according to swabs samples

Chi-square value	Swabs in NDP#		Swabs in DP^		Bacteria (primer)
	%	No./50	%	No./45	
8.261 **	56	28	84.44	38	*S. mutans* (*gtfD*)
7.893 **	54	27	77.77	35	*S. salivarius (gtfK)*
6.774 **	56	28	73.33	33	*S. oralis (gtfR)*
---	0.773 NS	---	4.381 *	---	Chi-square value

**: high significant difference (P<0.01 *: low significant difference (P<0.05 NS: no significant ^: Diabetic Patient #: Non-Diabetic Patient

Food debris could contribute for a greater occurrence of decays in diabetic patients. Specific types of acid-producing bacteria, especially *S. mutans*, colonize the dental surface and cause damage to the hard tooth structure in the presence of fermentable carbohydrates *e.g.* sucrose and fructose (Jaweda *et al.,* 2011).But other factors , such as lower sugar ingestion, could account for a lower occurrence rate (Amaral *et al.,* 2006). Saliva has been regarded as protective fluid against dental caries through its special properties and composition (Radhi, 2011). Poor glycemic control of diabetes has been associated with dryness of mouth due to salivary dysfunction predisposing to dental caries (Chen and Pan, 2007). Acidic saliva with low flow rate aggravates the process of tooth decay which can be considered as national diseases because of their high prevalence rates (Chu *et al.*, 2008; Sampaio *et al.*, 2011). Similar results are arrived by Bastos *et al.* (2011), who have found out that diabetes significantly affect oral tissues and it causes changes in the periodontal tissues, oral mucosa, salivary gland function, and oral neural function and increases the risk for caries. Also, other studies relate dental caries has been more prevalent and even severe in diabetic patients than non diabetics (Karjalainen, 2000 and Madden *et al.*, 2008). The *gtf* primer sets were also compared to sequenced microbial genes that were present in Genbank (www.ncbi.nlm.nih.gov) (Benson *et al.*, 2008). This comparison showed that amongst sequenced microbes, the primers are very specific for the target streptococcal *gtf* genes. These findings indicate that the *gtf* primers are very specific, giving *gtf* PCR product with multiple strains of the target streptococcal species, and no product with any other DNA was tested. All products were identified as being *gtf* product and all were from templates corresponding to the appropriate *gtf* primer set. Each *gtf* primer set was so specific that the samples of streptococcal species were identified with the primers *gtf* product (ANDREA, 2010) .

Sequencing of *gtf*s gene

The PCR products of the *gtf*s (*gtfD*, *gtfK*, and *gtfR*) regions gene were screened by sequencing of 12 samples for dental caries patients (6 of diabetic and 6 non-diabetic).Nine samples showed acceptable results according NCBI matching ,while 3 samples showed no results for an error in sequencing system. All results were directly compared with the bacterial reference *gtf*s gene sequence and were analyzed by searches using NCBI nucleotide blast in NCBI Database (http: NCBI Reference Sequence), software program Chromas and Mega version 5 software (Tamura *et al.,* 2011) (http://www. megasoftware.net) and aligned by clustal W (codons). Sequencing analyses *gtf*s gene was performed as described previously by Hoshino *et al.* (2004) and Eriko *et al.*(2009). The alignment of the *S. mutans* sequences showed 98% alignment for 32D and 37N samples and 99% for 25D and S.M patients, which is identical to the *gtfD* gene of the genbank *S. mutans* Lj23complet genome strain. These results indicate that the *S. mutans gtf* gene appears to be quite conserved, with very little diversity, and that the same *S. mutans* strain was present in all tested individuals. The results also suggest that the strain of *S. mutans* sequenced appears to be *S. mutans* Lj23complete genome as there was only 1-2% nucleotide difference and these differences could be accounted for by unresolved nucleotides present in each sequence.

Whereas, the alignment of the *S. salivarius* sequences showed that every person had one *gtf* gene sequence in their mouth that was 99% for 36D and 83N samples identical to the *gtfK* gene of the genbank *S. salivarius* 57.1.Chromosomal complete genome strain .The results also suggest that the strain of *S. salivarius* sequenced was similar to that of *S. salivarius* 57.1 complete genome as there was only 1% nucleotide difference and these differences could be accounted for by unresolved nucleotides present in each sequence. On the other hand, the alignment of the *S. oralis* sequences was showed that every person had one *gtf* gene sequence in their mouth that was 99% for 10D and 80N samples and 97% for 12D identical to the *gtfR* gene of the genbank *S. oralis* ATCC 35037 genomic scaffold SCAFFOLD1. whole genome shotgun sequence strain.The results also suggest that the strain of *S. oralis* sequenced was similar to that of *S. oralis* ATCC ATCC 35037 genomic scaffold SCAFFOLD1, whole genome shotgun sequence strain as there was only

1-3% nucleotide different and these differences could be accounted for the unresolved nucleotides present in each sequence. The sequencing analysis of *gtfs* gene (*gtfD*, *gtfK* and *gtfR*) is summarized in table (4). The results revealed that in diabetic patients the highest percentage of recorded mutations was in the *gtfR* gene. Whereas in NDP, the highest percentage of recorded mutations is in the *gtfK* gene. In *gtfR* gene all mutations were the substitution for DP and NDP. Nevertheless, in DP the mutations in *gtfk* and *gtfD* genes are distributed between substitution and insertion mutations without recording any type of deletion mutation. But, in NDP, mutations were distributed between three types of mutations (substitution, insertion and deletion).

Table (5) shows that silent and missense mutation's effect for Dp patients for the three genes *gtfD*, *gtfR* and *gtfK* were at the same percentage and higher than the frameshift mutations . On the other hand for NDP, the highest percentage of the effect of mutations was missense mutations as compared with the two other types of effects (silent and frameshift). DNA sequencing of PCR products confirmed that they had been amplified from the targeted *gtf* genes demonstrating high specificity for the oral streptococcal target species. Sequence analysis of *gtfD*, *gtfK* and *gtfR* summarized in table (6). These results indicate that the amplified product of PCR is originated from the *gtf* gene in the corresponding organism found in saliva and swabs samples.

TABLE 4 : Percentages of the types of mutations in *gtfs* genes in diabetic patients and non-diabetic patients

gtfR (*S.oralis*) NO.(%)	*gtfK* (*S.salivarius*) NO.(%)	*gtfD* (*S.mutans*) NO.(%)	Type of mutation	
10(100)	4(80)	4(66.6)	Substitution	
0	0	0	Deletion	DP
0	1(20)	2(33.3)	Insertion	
10(71)	5(38)	6(56.25)	Total	
4(100)	8(100)	5(62.5)	Substitution	NDP
0	0	1(12.5)	Deletion	
0	0	2(25)	Insertion	
4(28)	8(61)	8(57)	Total	
14	13	14	Total type of mutation	

DP: Diabetic patients, NDP: Non-diabetic patients

TABLE 5: Description the effects of mutations *gtfs* genes in diabetic patients and non-diabetic patients

Total NO. (%)	*gtfR* (*S. oralis*) NO. (%)	*gtfK* (*S. salivarius*) NO. (%)	*gtfD* (*S. mutans*) NO. (%)	Effect of mutation	
9(22)	4(40)	4(80)	1(16.6)	Silent	
9(22)	6(60)	0	3(50)	Missense	
3(7)	0	1(20)	2(33.3)	Frameshift	DP
21(51)	10(71)	5(38)	6(43)	Total	
4(9)	0	4(50)	0	Silent	NDP
13(31)	4(100)	4(50)	5	Missense	
3(7)	0	0	3	Frameshift	
20(48)	4(28.5)	8(62)	8	Total	
41(100)	14	13	14	Total Effect of mutation	

DP: Diabetic patients, NDP: Non-diabetic patients

TABLE 6: Analysis sequencing of *gtfD*, *gtfK* and *gtfR* in diabetic patient's samples and non-diabetic patient's samples

Effect in translation	Type of mutation	Site	Change in amino acid	Mutation type	Wilde type	Patients
Frameshift	Insertion	384	Insertion C	TAC	TA-G	*gtfD*
Missense	Substitution	387	Ala A/ GluE	GGG	GCG	25DP
Missense	Substitution	465	Lys K/ Arg E	CGA	AAA	*gtfD* 32
Missense	Substitution	471	Arg R/ Glu E	CAA	CAG	DP
Silent	Substitution	504	Arg R/ Glu E	GGG	CGG	
Frameshift	Insertion	507	Insertion G	GCC	G-CC	
Missense	Substitution	6	Asp D / Glu E	GAG	GAT	*gtfD* 37
Missense	Substitution	9	Asp D / TyrY	TAT	GAT	NDP
Missense	Substitution	15	Ala A / Gly G	GGG	GCG	
Frameshift	Dilation	27	Dilation A	-CC	AGC	
Missense	Substitution	357	Arg E / Ala A	GGA	AGA	
Frameshift	Insertion	384	Insertion G	GGG	GG-C	

Missense	Substitution	381	Ala A / Gly G	GGG	GCG	*gtfD* SM NDP
Frameshift	Insertion	387	Insertion C	CAT	C-AT	
Silent	Substitution	324	Repetitive	aaa	AAA	*gtfK* 36 DP
Silent	Substitution	327	sequence	aaa	AAA	
Silent	Substitution	330		gaa	GAA	
Silent	Substitution	333		aaa	AAA	
Frameshift	Insertion	426	Insertion A	AAA	AA-T	
Missense	Substitution	9	Asn R/ Phe F	TTC	AAC	*gtfK* 83 NDP
Missense	Substitution	15	Arg E / Lys K	AAA	AGA	
Missense	Substitution	21	Ser S/ Arg E	AGG	AGC	
Missense	Substitution	27	Ser S / Asn N	ATT	AGT	
Silent	Substitution	324	Repetitive	aaa	AAA	
Silent	Substitution	327	sequence	aaa	AAA	
Silent	Substitution	330		gaa	GAA	
Silent	Substitution	333		aaa	AAA	
Missense	Substitution	198	Phe F/ Leu L	CTC	TTC	*gtfR* 10 DP
Missense	Substitution	267	Ser S / Asn N	AAC	AGC	
Silent	Substitution	33	Leu L / Leu L	TTG	CTG	*gtfR* 12 DP
Missense	Substitution	45	Ili I / Lys K	AAA	ATA	
Missense	Substitution	48	Ser S / Asn N	AAC	AGT	
Missense	Substitution	156	Leu L / GluE	CAG	CTG	
Missense	Substitution	213	Thr Y / Asp D	AGC	ACC	
Silent	Substitution	216	Arg E / Arg E	AGA	AGG	
Silent	Substitution	228	GluE / GluE	CAG	CAA	
Silent	Substitution	279	Leu L/ Leu L	CTG	CTA	
Missense	Substitution	21	Arg E / Thr Y	AGG	AGA	*gtfR* 80 NDP
Missense	Substitution	42	Ala A/ Thr Y	ACC	GCC	
Missense	Substitution	126	Ili I/ Thr Y	ACT	ATT	
Missense	Substitution	303	GluE / Pro P	CAA	CCA	

DP: Diabetic patients NDP: Non-diabetic patients

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