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Comparison Between Traditional and PCR Analysis for Identification of Oral *Streptococci* with Dental Caries in Iraqi Diabetic Patients

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Abstract: The relationship between diabetic and non-diabetic patients were determined according to the dental caries occurrence and its causes by *Streptococcus* spp. (*Streptococcus mutans*, *Streptococcus salivarius* and *Streptococcus oralis*)which are isolated from oral cavity, In addition, this study was carried out to study the comparison between the traditional (bacterial culturing) and molecular diagnosis methods. The total number of the studied groups was 95 Iraqi patients ; 45 diabetic dental caries patients (DDCP) and 50 non-diabetic dental caries patients (NDCP) of both genders who their ages ranged from 18-65 years old. The patients, samples including saliva and buccal swabs that randomly collected from DDCP and NDCP who were reviewing Al-Alweyia Centers of Dental Caries and diabetic diseases in Al-Yarmook Hospital in Baghdad city. The results of culturing samples (saliva and buccal swabs) on mitissalivarius bacitracin agar media (MSBA) appeared that out of 95 bacterial cultures, 67 bacterial cultures were grown (32 bacterial cultures for diabetic dental caries patients and 35 bacterial cultures for non diabetic dental caries patients); *S. mutans*, *S. salivarius*, and *S. oralis* species were identified according to the results of microscopic examination, API 20-strep, hemolysis on blood agar, motility test and catalase test. The molecular study focused on the analysis of DNA which extracted directly from saliva, buccal swabs and from the bacterial culture cells of S. *mutans*, *S. salivares* and *S. oralis* from both diabetic dental caries patients and non diabetic dental caries patients. Polymerase chain reaction (PCR) results revealed the presence of the product with 433, 544, and 374 bp which were related to gtfD (*S. mutans*), gtfK (*S. salivarius*) and gtfR (*S. oralis)* respectively in all samples (saliva, buccal swabs and bacterial culture). According to the presence of these three genes, there were high significant differences at ($p<0.01$) between DDCP and NDCP, while there were no significant differences according to the percentage of presence of each gene between the three species of bacteria.

Keywords: Diabetic mellitus, Dental caries, Oral Streptococci spp., PCR technique, gtf gene.

مقارنة بين التحليل التقليدي وتقنية PCR لتشخيص بكتيريا المسبحيات الفموية في مرضى السكري العراقيين المصابين بتسوس الاسنان

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الخلاصة: لقد تم تحديد العلاقة بين مرضى السكري و مرضى غير السكري المصابين بتسوس الاسنان اعتمادا على تسوس الاسنان بانواع *Streptococcus* (*Streptococcus mutans* و *Streptococcus salivarius* و *Streptococcus oralis*) المعزولة من تجويف الفم، بالإضافة إلى دراسة العلاقة بين الطرق التقليدية (التنمية على الاوساط الزرعية) والطرق الجزيئية في تشخيص البكتريا. كان العدد الكلي للمجاميع المدروسة 95 مريض (45 مرضى السكري المصابين بتسوس الاسنان (DDCP) و 50 مرضى غير السكري المصابين بتسوس الاسنان (NDCP)) لكلا الجنسين الذين تتراوح أعمارهم بين 18–65 سنة. جمعت العينات من اللعاب ومسحات بطانة الفم الداخلية عشوائيا من مرضى السكري المصابين بتسوس الاسنان وغيرالسكري المصابين بتسوس الاسنان المراجعون لمركز آلعلوية التخصصي لطب الأسنان ومركز أمراض السكري في مستشفى اليرموك في مدينة بغداد.اظهرت نتائج عينات (اللعاب ومسحة بطانة الفم الداخلية) المزروعة على وسط المايتس ساليفارس بـ *ari* استراسين اكار (MSBA) ان من أصل 95 عينة مزروعة، نمت 67 بكتريا مزروعة (32 بكتريا مزروعة لمرضى السكري المصابين بتسوس الاسنان و 35 بكتريا مزروعة لمرضى غير السكري المصابين بتسوس الاسنان) ؛ شخصت الأنواع البكتيرية. *S. mutans* و *S. salivarius* و*S. oralis* اعتمادا على الفحص االمجهري، و اختبار API 20-strep وتحلل الدم على أكار الدم واختبار الكتليز. تركزت الدراسة الجزيئية على تحليل الحمض النووي الذي تم استخلاصه مباشرة من اللعاب ومسحات بطانة الفم الداخلية ومن خلايا البكتريا المزروعة (*S. mutans S. salivarius* و *S. oralis*) من كلا مرضى السكري المصابين بتسوس الاسنان والمرضى غير السكري المصابين بتسوس الاسنان. اظهر تفاعل البلمرة المتسلسل نواتج بحجم 433 و 544 و 374 زوج قاعدي التي تعود لجينات *gtfD (S. mutans)* و *gtfK (S. salivus)* و *gtfR* (*S. oralis*) على التوالي في جميع العينات (اللعاب ومسحات بطانة الفم الداخلية والزرع). اعتمادا على وجود هذه الجينات الثلاثة كانت هناك فروقات معنوية احصائية عالية ($p<0.01$) بين مرضى السكري المصابين بتسوس الاسنان مع مرضى غيرالسكري المصابين بتسوس الاسنان، بينما لم تكن هناك فروق معنوية إحصائية وفقا لنسبة وجود كل من الجينات بين الانواع الثلاث من البكتريا.

Introdaction

The oral cavity contains the greatest biodiversity. Over 70 species being isolated from mouth mucosa, saliva, denture surfaces and dental-plaque (1). The oral streptococci, representing over 80% of the mouth micro flora, are able to synthesize glucosyltransferases, enzymes involved in glucans production. Glucans are involved in the production of an extracellular slime layer promoting adhesion and formation of a dental plaque biofilm (2).

Diabetic mellitus is one of the serious systemic diseases that may cause general systemic changes, which may be reflected in the oral cavity (3). 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 (4). 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 (5).

Most oral streptococci possess glucosyltransferase (GTF) enzyme that use sucrose as a substrate to synthesize extracellular polysaccharide, which is an obligate factor for biofilm of dental plaque formation, and which facilitates the adhesion and accumulation of oral bacterial cells to tooth surfaces. Since both commensal and cariogenic streptococci have GTF enzymes, the proposed detection would help determine bacterial composition of the oral flora (6). 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 technique using species-specific primers (gtfD, gtfK and gtfR) which target almost the full length of the analyzed gene were applied to identify different bacterial cells species, these are S. mutans, S. salivarius, and S. oralis, respectively (7). The present study aims to differentiate different types of oral Streptococci which present in the mouth depending on gtfs gene using PCR technique as direct diagnostic method to identify the causative agent's which are associated with oral disease using species-specific primer and compression between the result of diagnosis of oral streptococci according to traditional (API 20-strep) and PCR method.

Materials and Methods

Samples Collection and Culturing

This study includes 95 samples (saliva and buccal swabs), which were randomly collected from human oral cavity during the period from November, 2012 to May, 2013 from

Al-Alweyia Center of Dental Caries and Center of Diabetic Diseases in Al-Yarmook hospital in Baghdad city. These subjects, included 45 patients (15males and 30 females) from diabetic dental caries patients (DDCP) and 50 samples (21males and 29 females) from non-diabetic dental caries patients (NDCP) for both genders with age ranged from (18-65) years old. Buccal swabs samples were put in sterile tubes containing 5ml sterilized 1X PBS (8).While; 1ml of saliva was put in sterile tubes. The samples were transported in ice box to the laboratory. All 95 samples (saliva and buccal swabs) were cultured on MSBA to inhibit growth of most types of bacteria except S. salivarius, S. mutans, and S. oralis.

Isolation and Identification of Oral Bacteria

Saliva samples were vortexed for 30s. and serially diluted with sterilized normal saline (1:10, 1:100 and 1:1000) until they reached to 10-3, then they were routinely cultured in treptic soy broth (TSB) with azid and cristal violate and incubating at 37°C for 24hrs, then 100µl from both samples saliva and buccal swabs were cultured on selective media on MSBA . The isolated colonies from both samples (saliva and buccal swabs) were sub cultured on MSBA agar plates and incubated at 37ºC for 48 hrs in un-aerobic jar as 5% CO2 enriched atmosphere (9).

Traditional Methods for Identification of Bacterial Samples

After estimation of positive samples on the surface of MSBA agar medium, small colonies were sub-cultured on the surface of blood-agar plates for further purification and incubated un-aerobically for two days at 37°C. The following methods were used for initial characterization of the isolates:

- Colonial shape and form on MSBA agar and blood agar.
- Gram-staining and microscopic examination.
- Catalase test.
- Motility test.
- Rapid API 20-Strep.

Genotyping

All subjects, samples (saliva and buccal swab), which were collected from oral cavity were transported to the laboratory immediately for culturing and extraction the total bacterial genomic DNA using genomic DNA extraction Mini kit (Geneaid, Korea), following the protocol provided by manufacturer. PCR technique was used to detect the gtfs genes using species-specific primers targeting almost the full length of the analyzed gene (Table1).

Table (1): Primer sequences used in this study

Target	Primer	Name Sequence of primers (BioNeer, Korea) 5'-3'	Product Size (bp)
S. mutants	gtfD F	5' GGCACCACAACATTGGGAAGCTCAGTT 3'	
	R	5' GGAATGGCCGTAAGCTAACAGGAT 3'	433
S. salivarius	gtfK F	5' GTGTTGCCACATCTTCACTCGCTTCGG 3'	
	R	5' CGTTGATGTGCTTGAAAGGGCACCATT 3'	544
S. oralis	gtfR F	5' TCCCGGTCAGCAAACTCCAGCC 3'	
	R	5' GCAACCTTTGGATTTGCAAC 3'	374

*F: Forward R: Reverse

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 with the following cycling parameters: an initial DNA denaturation at 95oC for 5 min was carried out for 1 cycle and then 38 PCR cycles consisting of denaturation at 94oC for 22 sec., annealing for 1 min. at (69, 68, 66oC) for (gtfD, gtfK, gtfR) respectively and extension at 72 oC for 37 sec. and final extension at 72 oC were carried out for 2 min. The PCR products were analyzed by electrophoresis in 2% agarose gel-TBE buffer. The gel was stained with ethidium bromide (0.5 µg/ml as final conc.) and photographed under UV illumination. A 100-bp DNA ladder (promiga, USA) was used as the molecular size standard.

Statistical Analysis

The Statistical Analysis System-SAS version 9.1(10) was used to reveal the effect of different factors percentage of study parameters. Chi-square test was used to show a significant comparison between percentages in this study.

Results and Discussion

Isolation and Identification of Strepococcus spp

The results of sample bacterial culturing on MSBA media appeared that from 95 samples, 67 bacterial cultures were grown including 32 bacterial cultures for DDCP and 35 bacterial cultures for NDCP. As it is commonly believed that DDCP with poorly controlled state and more aggressive dental caries have higher growth prevalence than NDCP . It is important point that the bacteria in the mouth of DDCP under stress condition were more than that in NDCP. This explains the appearance of higher bacterial growth was in DDCP samples.

Colony Characterization

S. mutans, S. salivarius, and S. oralis species were isolated on the surface of MSBA according to the results of API 20-strep kit, and hemolysis was identified according to the appearance on blood agar surface as shown in table 2. These results are in agreement with other studies (9,11,12 and 13).

Table (2): Characterization of isolated colony on culture medium

Bacteria	Blood agar	Mitis Salivarius Bacitracin Agar
S. mutans	varied between , hard coherent , raspberry like highly refractile , raised colonies circular, small, gray, domed, matte non hemolytic	Blue mucoid colonies with brown centers; adhere to agar raised, opaque, produce rough colonies that often look like frosted glass in appearance, convex may exhibit a glisting synthesis of glucan from sucrose.
S. salivarius	aminute circular, small, whit, domed, matte non hemolytic.	round shape convex large , diameter (2-5 mm) pale blue, and smooth or rough, mucoid colonies (gum-drop)
S. orails	firmly attached to agar circular, small, gray, matte non hemolytic	Blue, small, flat, hard colonies with a dome center.

The results of Microscopic examination indicated that the isolates were related to genus Strepococcus because they appeared as gram-positive spherical or ovoid cocci arranged in pairs and chains as well as they were non-spore forming and non-motile and give negative results for Catales test as confirmed by Macfaddin (14). These characterization are identical with Holt et al. (15); Brook et al. (16) and Whit-worthn et al.(17), and are related to S. mutans, S. salivarius, and S. oralis. The results of API 20-strep showed that S. mutans, S. salivarius, and S. oralis isolates have similar characterization to those obtained by AL-Sa'ady (13) and Ibtihal et al.(18) (Table-3).

Table (3): Results of API 20-Strep for S. mutans, S. salivarius, and S. oralis

Test	S. mutans	S. salivarus	S. oralis
VP	-	+	-
HIP	-	-	-
ESC	-	+	-
PYRA	-	-	-
α- GAL	-	-	-
β- GUR	V	+	V
β-GAL	-	-	-

PAL	-	-	V
LAP	+	+	+
ADH	-	-	-
RIB	-	-	-
ARA	-	-	-
MAN	+	-	-
SCR	+	-	-
LAC	+	+	+
TRE	+	V	V
INU	+	V	-
RAF	+	+	V
AMD	-	V	+
GLYG	-	-	-

(+) Positive result; (-) negative result; (V) variable results

PCR analysis for detection of gtfs gene

PCR amplified regions of gtfs gene gtfD, gtfK, and gtfR in S. mutans, S. salivarius and S. oralis are shown 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 buccal swabs) for DDCP and NDCP. (Fig.1, 2 and 3). Hoshino et al. (19) found similar sizes of the above amplified genes.

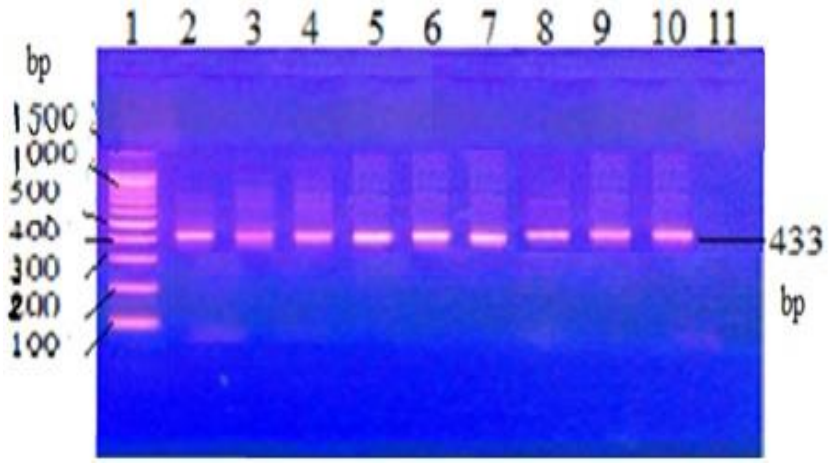

Figure (1): PCR product of gtfD (S. mutans) region 433 bp electrophoresed on 2% agarose gel 1volt/cm2. Lane 1: DNA ladder (100 bp), Lane 2,3,4,5, and 6 from DDCP samples Lane:7,8,9 and 10 from NDCP samples, Lane 11 :negative control. DDCP: diabetic dental caries patients; NDCP: non-diabetic dental caries patients.

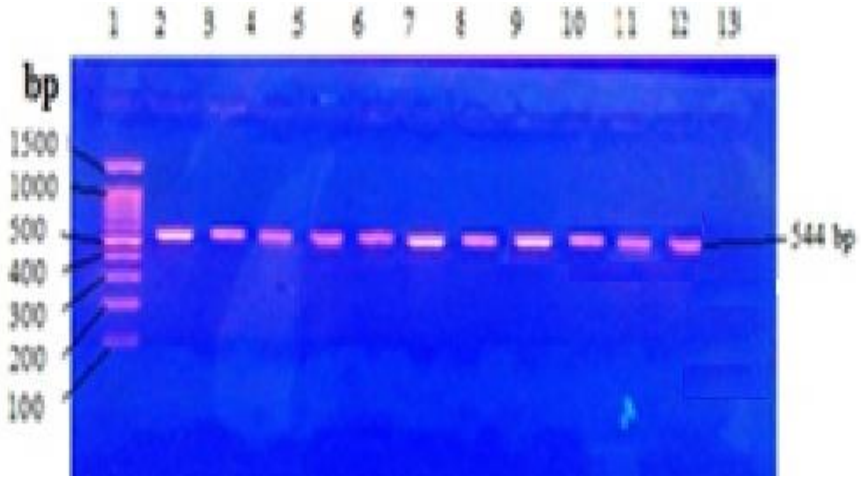

Figure (2): PCR product of gtfK (S. salivarius) region 544 bp electrophoresed on 2% agarose gel 1volt/cm2. Lane 1: DAN ladder (100 bp), Lane 2,3,4,5, and 6: PCR product from DDCP samples, Lane:7,8,9,10,11 and 12: PCR product from NDCP samples, Lane 13: negative control. DDCP: diabetic dental caries patients; NDCP: non-diabetic dental caries patients.

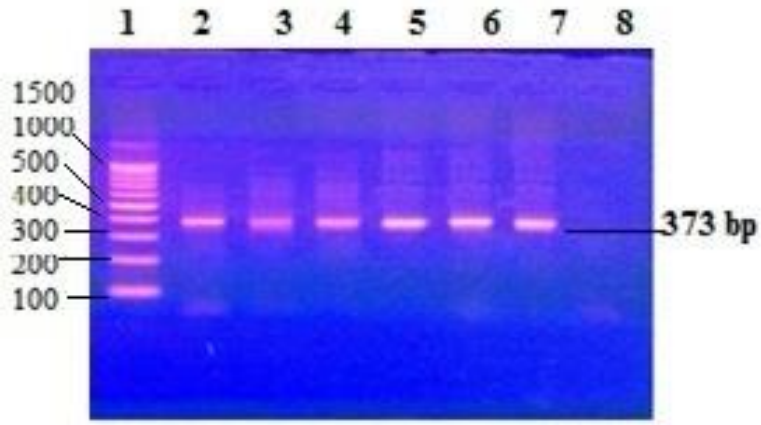

Figure (3): PCR product of gtfR (S. oralis) region 374 bp electrophoresed on 2% agarose gel 1volt/cm2. Lane 1: ladder Marker (100 bp), Lane 2,3 and 4: from DDCP samples 5,6 and 7: from NDCP samples, Lane 8: negative control. DDCP: diabetic dental caries patients; NDCP: non-diabetic dental caries patients.

PCR Analysis

Table (4) showed high significant difference at $p<0.01$ for the identification of gtfD, gtfK and gtfR for S. mutans, S. salivarius and S. oralis respectively in saliva samples from DDCP than NDCP and similar results were obtained in buccal swabs and culture samples (Table 5 and Table 6). This indicates that S. mutans, S. salivarius and S. oralis were more causative agent for dental caries. This comparison showed that amongst

sequenced microbes, the primers are very specific for the target Strepococcal gtf genes. 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 Strepococcal species were identified with the primers gtf product (20). Dental caries and dental plaque are among the most common diseases worldwide, and are caused by a mixture of microorganisms and many factors such as food debris could contribute for a greater occurrence of decays in DDCP. 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 (21), but other factors, such as lower sugar ingestion, could account for a lower occurrence rate (21). Saliva has been regarded as protective fluid against dental caries through its special properties and composition (23). Poor glycemic control of diabetes has been associated with dryness of mouth due to salivary dysfunction predisposing to dental caries (24). 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 (25). It is well known that a significant reduction of salivary flow leads to xerostomia which is the most common oral manifestation of diabetes. It is of paramount importance to inform and make diabetics aware of the beneficial properties of saliva, thus saliva's function of washing and cleaning the oral cavity is known to prevent the accumulation of plaque and debris. However, salivary glucose, acidity of the oral cavity, salivary viscosity, reduced salivary flow rate, and salivary gland dysfunction could be contributing factors in diabetic increased risk for periodontal disease and dental caries (26).

The relationship between dental caries and diabetes should be considered an important point when the long-term metabolic controls of the diabetes are taken into account. Similar results are arrived by Bastos et al. (27), 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 DDCP than NDCP (28). People with diabetes are more likely to develop periodontal infections, and tooth decay (29). Some studies did not find any association between the two diseases (decays and diabetes) (30), while other studies have found that DDCP with a poor metabolic control showed adverse outcomes regarding tooth decays index (31).

Table (4): Distribution of gtfD, gtfK and gtfR in diabetic and non-diabetic dental caries patients groups according to saliva samples

Bacteria (primer)	Saliva in DDCP		Saliva in NDCP		Chi-square value
	No. 45	%	No.50	%	
S. mutans (gtfD)	41	91.11	30	60	7.944 **
S. salivarius (gtfK)	38	84.44	27	54	8.364 **
S. oralis (gtfR)	39	86.66	29	58	8.509 **
Chi-square value	---	.278 NS	---	1.314 NS	---

** high significant difference (P<0.01) NS: no significant difference. DDCP: diabetic dental caries patients; NDCP: non-diabetic dental caries patients.

Table (5): Distribution of gtfD, gtfK and gtfR in diabetic and non-diabetic dental caries patients groups according to buccal swabs samples

Bacteria (primer)	Buccal swabs in DDCP		Buccal swabs in NDCP		Chi-square value
	No. 45	%	No. 50	%	
S. mutans (gtfD)	38	84.44	28	56	8.261 **
S. salivarius (gtfK)	35	77.77	27	54	7.893 **
S. oralis (gtfR)	33	73.33	28	56	6.774 **
Chi-square value	---	4.381 *	---	0.773 NS	---

** high significant difference (P<0.01 *: low significant difference (P<0.05 NS: no significant difference. DDCP: diabetic dental caries patients; NDCP: non-diabetic dental caries patients.

Table (6): Distribution of gtfD, gtfK and gtfR in diabetic and non-diabetic dental caries patients groups according to culture samples

Bacteria (primer)	Culture in DDCP^		Culture in NDCP#		Chi-square value
	No. 45		No. 50	%	
S. mutans (gtfD)	22	68.75	17	48.57	6.845 **
S. salivarius (gtfK)	19	59.37	15	42.85	6.134 **
S. oralis (gtfR)	20	62.50	17	48.57	6.835 **
Chi-square value	---	3.982 *	---	0.429 NS	---

**: high significant difference (P<0.01 *: low significant difference (P<0.05 NS: no significant difference. DDCP: diabetic dental caries patients; NDCP: non-diabetic dental caries patients.

Comparison between the results of diagnosis according to traditional (API 20-Strep) and PCR methods

Table (7) shows that the percentage of bacterial culture samples that give identified S. mutans, S. saliverius and S. oralis according to PCR was higher significantly at (p<0.01) for DDCP than NDCP. On the other hand, this table demonstrates that the percentage of bacterial culture samples isolates using PCR method is higher significantly at (p<0.01) than using the traditional methods. Bacterial

culture methods were common methods for identification and characterization of pathogenic bacteria from the oral cavity as conventional methods. Bacterial Culture-based methods are widely accepted because they are relatively easy to be used, cost less, and demonstrated relationship to health risk (32). However, culture techniques were limited in their sensitivity and specificity, and the time required for sample processing ranges from 18 to 96 hrs, with confirmation and verification steps which take even longer time (33 and 34) .While, development technology provides new opportunities to detect bacteria more rapidly (32). Molecular biology methods have been developed to overcome culture problems. Therefore, the PCR was used in bacterial identification in environmental and clinical specimens. It was more sensitive, specific and faster than culture-based conventional methods in bacterial determination. It allows the detection of viable and non viable microorganisms; hence it directly measures DNA, and consumes less time and effort than conventional methods (35).

Thus, PCR-based methods are promising; their results may differ from those of the conventional culture-based methods that they are intended to be replaced. Since PCR measures genetic material rather than the viable cells quantified by culture-based methods, it may overestimate oral indicator bacteria because of the inclusion of target DNA from dead or dying cells in the measurement.

Conclusion

PCR method was more rapidly, sensitive, specific, hence, it directly measure DNA, and allow the detection of the viable and non viable micro-organisms in diagnosis the bacterial isolates than the traditional (API 20-Strep) methods in both study groups and direct detection of oral streptococci in saliva samples by using gtfs genes.

Table (7): Percentage of isolates from culture samples according to API 20-strep and PCR methodes

Bacteria	Diagnostic by API 20-strep				Diagnostic by PCR				Chi-square value
	DDCP		NDCP		DDCP		NDCP		
	No.32	%	No.35	%	No.32	%	No.35	%	
S. mutans	10	31.25	9	25.71	15	46.87	13	37.14	7.634**
S.salivarius	12	37.50	6	17.14	18	56.25	14	40.00	11.26**
S. oralis	14	43.75	8	22.85	17	53.12	14	40.00	9.802**
Chi-square value	---	4.83 *	---	1.15 NS	---	4.085 *	---	0.781 NS	----

**: high significant difference (P<0.01) *: low significant difference (P<0.05) NS: no significant difference. DDCP: diabetic dental caries patients; NDCP: non-diabetic dental caries patients.

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