Determinants of periodontopathogens in microbiological monitoring of diabetic patients with periodontitis

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ABSTRACT

الأهداف: تحديد وتحليل مدى انتشار الإصابة بجراثيم اللثة وذلك عن طريق المراقبة المجهرية الحيوية لمرضى السكري المصابين بالتهاب اللثة.

الطريقة: شملت هذه الدراسة المقطعية 352 من مرضى السكري المصابين بالتهاب اللثة، وكان هؤلاء المرضى قد توزع دخولهم على المستشفيات التالية: مستشفى القوات المسلحة بالرياض، ومستشفى الملك فيصل التخصصي ومركز الأبحاث، ومدينة الملك عبدالعزيز الطبية، ومستشفى القوات المسلحة بقاعدة الملك عبدالعزيز البحرية، ومدينة الأمير سلطان بن عبدالعزيز للخدمات الإنسانية، الرياض، المملكة العربية السعودية وذلك خلال الفترة من يوليو 2004م إلى أغسطس 2008م. لقد تم الاستعانة بتقنية التفاعل المبلمر (PCR) أثناء التحليل المجهري الحيوي لأنواع الجراثيم التالية: باكترويدس فورسيذاس (*Bacteroides forsythus*)، وأجريجاتباكتر أكتنوميسيتمكوميتانس (*Aggregatibacter actinomycetemcomitans*)، وبورفيروموناس جينجيفالس (*Porphyromonas gingivalis*)، وبريفوتيلا إنترميديا (*Prevotella intermedia*).

النتائج: لقد كان متوسط عمر المرضى 54.4 ± 0.67 (يتراوح ما بين 21 إلى 80 عاماً)، وبلغ عدد المرضى الذكور 214 مريضاً (61%)، فيما كان عدد الإناث 138 مريضة (39%). كان 36 من المرضى (10%) يعاني من مرض السكري من النوع الأول و 316 مريضاً يعاني من السكري من النوع الثاني (90%). أظهرت النتائج بأن 55.6% من المرضى كان مُصاباً بجرثومة باكترويدس فورسيذاس، و51.7% مصاباً بجرثومة أجريجاتباكتر أكتنوميسيتمكوميتانس، و63.7% مُصاباً بجرثومة بورفيروموناس جينجيفالس، و6.1% مُصاباً بجرثومة بريفوتيلا إنترميديا. ولقد كانت نسبة الإصابة بجراثيم اللثة أعلى في الذكور منها في الإناث وذلك في جميع الفئات العمرية. يزداد خطر الإصابة بجرثومة باكترويدس فورسيذاس في الفئة العمرية 50-41 عاماً، أما أجريجاتباكتر أكتنوميسيتمكوميتانس فتكون سائدة في الفئة العمرية 60-51 عاماً، وبورفيروموناس جينجيفالس في الفئة العمرية 60-51 عاماً، وبريفوتيلا إنترميديا في الفئة العمرية 40-31 عاماً.

خاتمة: تشير نتائج الدراسة بأن نسبة انتشار جراثيم باكترويدس فورسيذاس، وأجريجاتباكتر أكتنوميسيتمكوميتانس، وبورفيروموناس جينجيفالس كانت أعلى من نسبة انتشار جرثومة بريفوتيلا إنترميديا وذلك في مرضى السكري الذين يعانون من التهابات اللثة.

Objectives: To determine and analyze the frequency of periodontopathogens in microbiological monitoring of diabetic patients with periodontitis.

Methods: This cross-sectional study included 352 diabetic patients with periodontitis who were registered at Riyadh Armed Forces Hospital, King Faisal Specialist Hospital and Research Centre, King Abdul Aziz Medical City, Naval Base Hospital, and Sultan Bin Adulaziz Humanitarian City, Riyadh, Kingdom of Saudi Arabia from July 2004 to August 2008. Microbiological analysis comprised the detection of *Bacteroides forsythus* (*Bf*), *Aggregatibacter actinomycetemcomitans* (*Aa*), *Porphyromonas gingivalis* (*Pg*), and *Prevotella intermedia* (*Pi*) by polymerase chain reaction method.

Results: The mean age of patients was 54.4 ± 0.67 (range: 21-80 years). There were 214 (61%) males and 138 (39%) females. Among the study population, 36 (10%) had type 1, and 316 (90%) patients had type 2 diabetes. The results showed that 55.6% of patients had *Bf*, 51.7% had *Aa*, 63.7% had *Pg*, and 6.1% had *Pi*. The frequencies of periodontopathogens were higher in males than females in all age groups. The risk of periodontopathogens *Bf* were found higher level in 41-50 age group, *Aa* in 51-60, *Pg* in 51-60, and *Pi* in 31-40 age groups.

Conclusion: This study found that the frequencies of periodontal pathogens *Bf*, *Aa*, and *Pg* were higher than *Pi* in diabetic patients with periodontitis.

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The World Health Organization (WHO) and International Diabetes Federation (IDF) have predicted that the number of diabetics would increase significantly by the year 2030 to approximately 366 million, an increase of 214% compared to 2006.[1] Diabetes is associated with several complications, and some types have been linked to chronic hyperglycemic state. It is also frequently associated with pathological changes in the blood vessel walls.[2] Epidemiological studies have shown diabetes to be a risk factor for periodontitis, and periodontal disease is the sixth leading complication of diabetes.[3] Hyperglycemia appears to trigger a series of events leading to a higher risk of infections.[4,5] The association between diabetes and the increased susceptibility to oral infection including periodontal disease is significant.[6] People with diabetes who have periodontal disease have a harder time maintaining healthy blood glucose levels, and the macrophages play an important role in both diabetes and periodontitis.[6] Chronic periodontitis is a slowly progressing disease that is primarily the result of an inflammatory response to plaque and calculus accumulation.[7] More rapidly progressing clinical presentation of chronic periodontitis has been described in diabetic patients.[8,9] Several studies[10-12] have reported that bacteria involved in periodontitis are usually anaerobic Gram-negative bacteria. The pathogenic effect of Gram-negative bacteria, which causes direct and indirect damage to the periodontal supporting tissues via its toxic products, and activation of a series of inflammatory reactions is well documented.[10,13] Studies in both humans and animals have shown that periodontal inflammation and destruction of periodontal tissues are initiated, and supported by bacteria of dental plaque.[10] The association between oral diseases and the oral microbiota is well established. Of the more than 750 species of bacteria that inhabit the oral cavity, a number are implicated in oral diseases.[13] Of these species, only a small number are suspected periodontal pathogens.[14,15] Aggressive periodontitis has been postulated to be frequently associated with *Aggregatibacter actinomycetemcomitans* (*Aa*) and *Porphyromonas gingivalis* (*Pg*), *Bacteroides forsythus* (*Bf*) and *Prevotella intermedia* (*Pi*).[14-17] The aim of the present study was to determine and analyze the frequencies of periodontopathogens *Bf, Aa, Pg,* and *Pi* in microbiological monitoring of diabetic patients with periodontitis.

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Methods. This cross-sectional study included 352 diabetic patients with periodontitis who were registered at Riyadh Armed Forces Hospital, King Faisal Specialist Hospital and Research Centre, King Abdul Aziz Medical City, Naval Base Hospital, and Sultan Bin Adulaziz Humanitarian City, Riyadh, Kingdom of Saudi Arabia from July 2004 to August 2008. The inclusion criteria were as follows: age range between 21-80 years; diabetes identified as type 1 or 2; diabetes diagnosed ≥1 year, and good physical condition with no serious medical conditions or transmittable diseases such as, malignant disease; active hepatitis; have at least 6 periodontal sites in at least 2 different quadrants with probing pocket depth ≥5mm. The exclusion criteria were: no serious medical conditions or transmittable diseases such as, malignant disease; active hepatitis; no treatment with SRP (scaling and root planing) in the 6 months prior to the study; no use of antibiotics within 3 months prior to the study; and female patients not pregnant or nursing. The study was approved by the Research and Ethics Committee of Sultan Bin Abdulaziz Humanitarian City.

Periodontopathogen analysis. Bacterial analysis for pathogenic anaerobes: *Bf, Aa, Pg* and *Pi* were performed by polymerase chain reaction (PCR). For each patient, 4 sites with pocket depths of ≥5 mm were selected. The Florida probes were wiped with 70% isopropyl alcohol wipes between measurements to reduce bacterial contamination of the sites.[18] After the clinical measurements were recorded, a subgingival plaque sample was taken from each site using separate sterile curettes, and a single vertical stroke. Each sample was immediately placed in a sterile microcentrifuge tube containing 0.5 ml Tris ethylenediamine tetraacetic acid (EDTA [TE]) buffer (10 mM Tris hydrogen chloride [HCl] [pH 7.6], 1 mM EDTA [pH 8.0]). For PCR analysis, 90 ml of vortex-mixed subgingival plaque was added to 10 µl of 10 x lysis buffer (100 mM Tris-HC,l pH 8.0, 10 mM EDTA, 10% Triton X-100), and boiled for 5 min, then 5 µl of this lysate was used in each PCR reaction. The primers used for various PCR analyses are shown in **Table 1.**[19-23] The PCR amplification was carried out in a reaction volume of 25 µl consisting of 5 µl

Table 1 - Primers for PCR-based identification of periodontopathogens.[19-23]

Periodontopathogens	Primers sequence
Bacteroides forsythus	GCG TAT GTA ACC TGC CCG CA TGC TTC AGT GTC AGT TAT ACCT
Aggregatibacter actinomycetemcomitans	AGA GTT TGA TCC TGG CTG AG CAC TTA AAG GTC CGC CTA CGT GCC
Porphyromonas gingivalis	TGT AGA TGA CTG ATG GTG AAA ACC ACG TCA TCC CCA CCT TCC TC
Prevotella intermedia	TTT GTT GGG GAG TAA AGC GGG TCA ACA TCT CTG TAT CCT GCG T

sample lysate, and 20 µl of reaction mixture containing 1 x PCR buffer (10 mM Tris-HCl, pH 8.8, 1.5 mM magnesium chloride, 50 mM potassium chloride, 0.1% Triton X-100), 2 units of Taq DNA polymerase (Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA), 0.2 mM deoxynucleotide triphosphates, and 100 pmol of each primer. The PCR cycling was carried out in a DNA thermal cycler PE 480 (PerkinElmer, Covina, California, USA). The cycling conditions for *Bf*, *Aa*, and *Pg* were initial denaturation for 5 min at 95°C, 35 amplification cycles of denaturation at 95°C for one minute, annealing of primers at 60°C for one minute, and primer extension at 72°C for 1.5 minutes, followed by a final extension step at 72°C for 7 minutes. The cycling conditions for *Pi* were the same except that the annealing temperature was 58°C. The reaction products were analyzed immediately. Negative controls were included in each batch of samples being analyzed by PCR. Ten µl of each reaction product was fractionated on a 1.5% agarose gel containing ethidium bromide (0.5 mg/ml), using 50 or 100 bp DNA ladder (Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA) as a size marker, and visualized and photographed using gel documentation and analysis system (Ultra-Violet Products Ltd, Cambridge, England).

Data analysis was carried out using Microsoft Excel 2002 (Microsoft Corporation, Seattle, WA, USA) and GraphPad InStat Version 3 (GraphPad Software, San Diego, USA). The frequencies of *Bf, Pg, Pi* and *Aa* were presented as percentage. Age and gender wise frequencies of the periodontopathogens, and comparisons among the groups were performed by Fisher's exact test. A $p<0.05$ was considered significant.

Results. Age and gender wise distribution of the 352 diabetic patients with periodontitis included in the study are shown in **Table 2**. The mean age of patients was 54.4 ± 0.67 (range: 21-80 years). There were 214 (61%) males and 138 (39%) females. Among the study population, 36 (10%) had type 1, and 316 (90%) patients had type 2 diabetes. **Table 3** shows the age wise frequencies of the periodontopathogens in the study population. **Table 4** shows the gender wise frequencies of the periodontopathogens in the study population. The results showed that the frequencies of periodontopathogens were higher in males than females in all age groups. **Figure 1** shows the overall frequencies of periodontopathogens in the study population.

Table 2 - Age and gender wise distribution of the study population.

Age group, years	Age, mean ± SD	Gender		Type of diabetes	
		Male	Female	Type 1	Type 2
21-30	24.4 ± 1.3	14	9	5	18
31-40	36.3 ± 1.5	32	23	6	49
41-50	46.2 ± 0.3	55	41	9	87
51-60	55.2 ± 0.26	65	47	11	101
61-70	64.4 ± 0.37	39	11	4	46
71-80	75.1 ± 1.4	9	7	1	15
All groups	54.4 ± 0.67	214	138	36	316

SD - standard deviation

Discussion. It is generally accepted that the etiology of periodontitis is polymicrobial in nature, and periodontal disease is more common in individuals with diabetes mellitus (DM).[24-26] Worsening or improvement of periodontal status is accompanied by a shift in the bacterial composition of subgingival plaque.[24] The microbial testing can be used for analysis and to optimize periodontal therapy, and assess its outcome, especially when the treatment with antimicrobial drugs is considered.[14,27] The influence of diabetes on the onset and development of periodontal disease has long been

Table 3 - Age wise frequencies of periodontopathogens.

Age group, years	*Bf*	*Aa*	*Pg*	*Pi*
	(%)			
21-30	(47.7)	(34.69)	(47.7)	(10.8)
31-40	(56.3)	(39.9)	(65.3)	(13.0)
41-50	(64.5)	(56.3) ^{f}	(65.3)	(11.4)
51-60	(60.6)	(61.6)¶•	(66.8)	(7.0)
61-70	(48.0)	(48.0)	(66.0)	(2.0)
71-80	(24.9)*#†	(31.2)‡	(50.0)	(0.0)

p-values versus different age groups, Fisher's exact test. age group; *Bacteroides forsythus (Bf)* - *31-40 versus 71-80 (p=0.045), #41-50 versus 71-80 (p=0.0045), †51-60 versus 71-80 (p=0.013), *Aggregatibacter actinomycetemcomitans (Aa)*-¶21-30 versus 51-60 (p=0.021), ^{f}31-40 versus 41-50 (p=0.044), •31-40 versus 51-60 (p=0.012), ‡51-60 versus 71-80 (p=0.029), *Pg - Porphyromonas gingivalis, Pi - Prevotella intermedia*

Table 4 - Gender wise frequencies of periodontopathogens.

Age group, years	Gender	*Bf*	*Aa*	*Pg*	*Pi*
		(%)			
21-30	Male	(39.1)	(26)	(39.1)	(9.0)
	Female	(8.7)*	(8.7)	(8.7)^{f}	(1.8)
31-40	Male	(32.7)	(23.6)	(36.3)	(8.7)
	Female	(23.6)	(16.3)	(29)	(4.3)
41-50	Male	(39.5)	(33.3)	(34.3)	(6.2)
	Female	(25)#	(23.0)	(31)	(5.2)
51-60	Male	(34.8)	(36.6)	(39.2)	(5.3)
	Female	(25.8)	(25.0)	(27.6)	(1.7)
61-70	Male	(34.0)	(34.0)	(52)	(2.0)
	Female	(14.0)†	(14.0)‡	(14)¶	(0.0)
71-80	Male	(18.7)	(25.0)	(37.5)	(0.0)
	Female	(6.2)	(6.2)	(12.5)	(0.0)

Bf - Bacteroides forsythus, Aa - Aggregatibacter actinomycetemcomitans, Pg - Porphyromonas gingivalis, Pi - Prevotella intermedia. *p*-values male versus female, Fisher's exact test. Age groups: *Bf* - *21-30 (p=0.035), #41-50 (p=0.044), †61-70 (p=0.033), *Aa* - ‡61-70 (p=0.03), *Pg* - ^{f}21-30 (p=0.035), ¶61-70 (p=0.001).

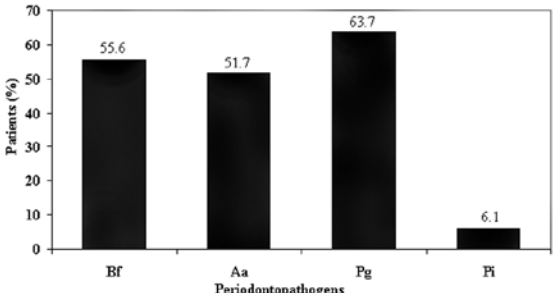

Figure 1 - The overall frequencies of periodontopathogens in the study population. *Bf - Bacteroides forsythus, Aa - Aggregatibacter actinomycetemcomitans, Pg - Porphyromonas gingivalis, Pi - Prevotella intermedia*

discussed with contradictory conclusions.[9,28-30] However, numerous studies have been carried out to examine the microflora of patients with DM. Conversely, results have been rather inconsistent.[17,26,29,31] Recent studies have shown that high levels of *Bf*, Aa and *Pg* presents in periodontal pockets of patients with DM.[17,29,31] On the other hand, no significant relationship was found between the prevalence of periodontopathogens and diabetic factors in diabetic patients.[32,33] It is possible that other factors may be more significant in the increased incidence and severity of periodontal diseases in poorly controlled diabetics.[26] In the present study, we found higher levels of *Bf*, *Aa,* and *Pg* among diabetic patients with periodontitis. Studies reported that more diabetic patients had higher levels of periodontopathogens than non-diabetic patients.[29,34] Bacterial products, such as histolytic enzymes, endotoxins, and exotoxins might exert a pronounced pathogenic effect. In addition, the reduced phagocytosis, leukotaxis, and leucocyte index have been reported in neutrophils from diabetics.[35]

Periodontal diseases in relation to various diabetic factors have been studied quite intensively, but less attention has been paid on the role of age. Studies reported that age was significantly associated with chronic periodontitis.[17,36,37] Thorstensson et al[36] compared diabetic patients and control in a 10-year-age subgroups among 40 and 70 years, and they reported that periodontal disease began earlier in diabetics than in controls, and the differences were most evident in the age group of 40-49 years, whereas another study[37] has suggested that high incidence of gingivitis occurred within the first 29 years with a cluster of cases between 10 and 29 years of age, and the incidence tend to decline with advancing age. However, the incidence of periodontitis was highest among adults over 40 years.[37] In addition, a study[38] reported that periodontopathogen were recovered in diabetics, as well as in non-diabetics. However, significantly more periodontopathogen were found in different age groups of diabetics compared to non-diabetics.[38] In contrast to the above studies, Yuan et al[39] reported no significant differences in age, gender, and prevalence of the periodontopathogens between the diabetic and non-diabetic groups. In this present study, we found that the risk of periodontopathogens *Bf* was higher in the 41-50 age group, *Aa* in 51-60, *Pg* in 51-60, and *Pi* in 31-40 age groups.

Several studies[17,40] reported that the incidence of periodontitis is 2-3 fold higher in diabetic patients than in non-diabetic, and among males the presence of periodontal pathogens were positively associated with periodontitis.The results of this present study showed that the frequencies of periodontopathogens were higher in males than females in all age groups. It should be noted here that the distribution of periodontal pathogens differs in various geographic locations and racial/ethnic groups.[31] The periodontal pathogens and several cytokines stimulate osteoclast differentiation in gingival connective tissue. Further, alveolar bone resorption progresses and the resultant tooth loss falls oral functions.[40] The study[40] also reported that periodontitis affected the diabetic state, in which periodontal pathogen and cytokines probably elevated the insulin resistance by inhibiting glucose incorporation into the smooth muscle cells.

The major limitation of this study was the limited number of bacteria examined. Further research is needed to address the limitations indicated in the study. Despite the limitation, the study provides valuable data for periodontal pathogens in diabetics patients. In addition, this type of study among diabetic patients with periodontitis might provide new information and understanding among the Saudi population.

In conclusion, the findings of the present study indicate that the frequencies of periodontal pathogens *Bf, Aa* and *Pg* were higher than *Pi* in diabetic patients with periodontitis. However, further studies are required in different clinical settings to provide a more comprehensive picture of periodontopathogens in diabetic's patients.

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