Prevalence of Periodontal Pathogens and Metabolic Control of Type 1 Diabetes Patients

Rok Schara DDS, PhD[1], Eva Skaleric DDS, PhD[1], Katja Seme MD, PhD[2] and Uros Skaleric DDS, PhD[1]

[1]Department of Oral Medicine and Periodontology, and [2]Institute for Microbiology and Immunology, Medical Faculty, University of Ljubljana, Slovenia

Abstract

Objective: The aim of the study was to evaluate the prevalence of periodontal pathogens *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia* and *Treponema denticola* in subgingival plaque collected at different probing depths of type 1 diabetes patients with periodontal disease in correlation to metabolic control. **Methods:** Twenty-one patients 40 to 50 years old were included in the study. In each patient blood samples were taken for the evaluation of HbA1c level and subgingival plaque samples were taken with paper points from the two deepest pockets. The presence of five periodontal pathogens was detected by multiplex polymerase chain reaction. **Results:** The results of 38 plaque samples showed that *T. forsythia* was found in 48% followed by *T. denticola* in 31%, *P. gingivalis* in 26%, *P. intermedia* in 9% and *A. actinomycetemcomitans* in 7%. *T. forsythia* and *T. denticola* were the most frequent combination of periodontal pathogens found in the same sample. The serum level of HbA1c in nine patients where *T. forsythia* was detected (7.5 ± 1.4%) was significantly higher (F-test, $p = 0.001$) than in 12 patients where *T. forsythia* was not detected (6.8 ± 0.5%). Similarly, the serum level of HbA1c was significantly higher (F-test, $p = 0.001$) in eight patients where *T. denticola* was detected (7.5 ± 1.8%) compared to the 13 patients where *T. denticola* was not detected (7.0 ± 0.5%). No such correlation was found for *P. gingivalis, P. intermedia* or *A. actinomycetemcomitans*. **Conclusions:** We conclude that *T. forsythia* and *T. denticola* are most frequently found in subgingival plaque samples of type 1 diabetic patients and these findings correlate with poorer metabolic control of diabetes.

Key words: Periodontology, microbiology, diabetes type 1, HbA1c

Introduction

Diabetes is a systemic disease with a number of major complications that may adversely affect quality and length of life. The disease is characterized by an increased susceptibility to infection, poor wound healing, and increased morbidity and mortality associated with disease progression (Williams, 2008). Periodontal disease has been reported by Loe as the sixth complication of diabetes, along with neuropathy, nephropathy, retinopathy, and micro- and macrovascular diseases (Loe, 1993). Diabetes mellitus not only increases the risk for and prevalence of periodontal disease, but it also augments the progression of periodontal destruction. The mechanisms by which periodontal disease may affect the diabetic state have been elucidated only recently (Genco *et al.*, 2005; Nishimura *et al.*, 2003). Both periodontal disease and diabetes have major inflammatory components. Studies have demonstrated the influence of periodontal pathogens on diabetes mellitus (Ainamo *et al.*, 1990; Tervonen *et al.*, 1994; Thorstensson *et al.*, 1995). Systemic bacterial and viral infections such as the common cold or influenza result in increased systemic inflammation, which increases insulin resistance and makes it difficult for patients to control blood glucose levels (Mealey and Oates, 2006). Chronic periodontal disease also has the potential to exacerbate insulin resistance and worsen glycemic control, while periodontal treatment decreases inflammation and may help diminish insulin resistance (Nishimura *et al.*, 2000). A study by Sastrowijoto *et al.* (1990) showed periodontal pathogens at a higher level

Correspondence to: Rok Schara
Department of Oral Medicine and Periodontology, Medical Faculty, University of Ljubljana,
Hrvatski trg 6, 1000 Ljubljana, Slovenia
Telephone: +38640293614 Fax: +38615222494
email: rok.schara@mf.uni-lj.si

in patients with poor metabolic control. The risk of developing periodontal disease (Soskolne, 1998) may be greater in patients with diabetes who have poor glycemic control than in patients with well-controlled diabetes (Grossi and Genco, 1998; Takeda *et al.*, 2006). Studies have provided evidence that control of periodontal infection has an impact on improvement of glycemic control (Grossi, 2001; Vergnes *et al.*, 2009), evidenced by a decrease in demand for insulin and decreased HbA1c levels (Mealey and Rethman, 2003). Several studies suggest that patients with periodontal disease, particularly those colonized with Gram-negative bacteria such as *Porphyromonas. gingivalis*, *Tannerella forsythia* and *Prevotella intermedia*, have higher levels of inflammatory serum markers such as C-reactive protein (CRP), IL-6, and fibrinogen than patients without periodontal disease. Similarly, there is an increase in resistance to insulin, decreasing glycemic control (Lalla *et al.*, 2007; Nibali *et al.*, 2007; Nishimura *et al.*, 2000; Offenbacher *et al.*, 1997).

The aim of our study was to evaluate the presence of some periodontal pathogens in subgingival plaque samples from pockets of different probing depth and to correlate these findings with the level of metabolic control of type 1 diabetes patients.

Patients and methods

Twenty-one patients participated in our study, 17 females and four males, recruited from the Department of Endocrinology, Diabetes and Metabolic Diseases of the University Medical Center Ljubljana, with diagnosed type 1 diabetes mellitus. The age range was 40 – 50 years (mean 44 ± 4 years). The duration of diabetes was 10 – 40 years (mean 23 ± 7 years). The serum level of HbA1c, indicating the level of metabolic control, was found to be in the range of 6.2 - 9.2% (mean 7.2 ± 0.7%).

The patients were without systemic inflammatory complications and without a history of antibiotic therapy or non-steroidal anti-inflammatory drug therapy for three months before the first visit. To be included in the study they had to have at least 20 teeth in the functional dentition, excluding third molars. They had to have a clinical diagnosis of periodontal disease as determined by the presence of four teeth in at least two quadrants with 5 mm periodontal pockets and bleeding on probing. Clinical parameters included: plaque index (PI), bleeding upon probing, probing depth and clinical attachment level. All parameters were recorded at six measuring points (mesiobuccal, buccal, distobuccal, mesiolingual, lingual, and distolingual) on all teeth (excluding third molars).

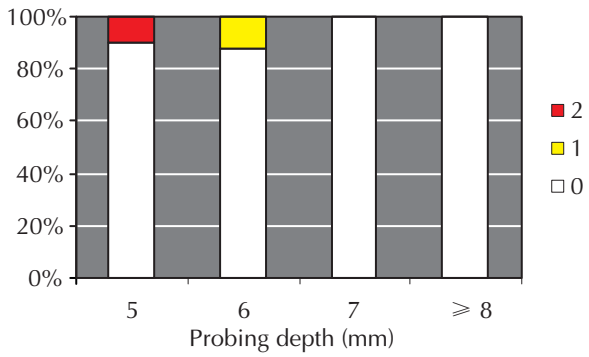
Blood samples were taken for the evaluation of HbA1c level. Periodontal pathogens were evaluated in subgingival plaque samples taken with paper points (Jervoe-Storm *et al.*, 2007) from the two deepest pockets in each patient. Each periodontal pocket was sampled by one sterile paper point, which was inserted to the depth of the pocket, kept in place for 10 s and then transferred to a sterile 1.5 mL microtube. Each sampling site was tested for the presence of *Aggregatibacter actinomycetemcomitans, P. gingivalis, P. intermedia, T. forsythia* and *Treponema denticola* by a multiplex polymerase chain reaction (PCR), followed by hybridization against species-specific DNA probes using a micro-IDent test (Hain Lifescience, Nehren, Germany) which was performed following the manufacturer's instructions. Briefly, the DNA was extracted using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. PCR amplification was carried out in a reaction volume of 25 µL consisting of 2.5 µL of template DNA and 22.5 µL of reaction mixture containing 17.5 µL of primer–nucleotide mix from the micro-IDent kit, 2.5 µL of 10× PCR buffer, 2.5 µL of 25 mmol/L $MgCl_2$, and 1 U FastStart Taq polymerase (Roche Diagnostics, Mannheim, Germany). PCR cycling was carried out in a Mastercycler Personal (Eppendorf, Hamburg, Germany). The cycling conditions comprised an initial denaturation step at 95°C for 5 min; 10 cycles at 95°C for 30 s and at 58°C for 2 min; 20 cycles at 95°C for 25 s, at 53°C for 40 s, and at 70°C for 40 s; and a final extension step at 70°C for 8 min. According to the manufacturer's instructions, for the subsequent reverse hybridization the biotinylated amplicons were denatured and incubated at 45°C with hybridization buffer and strips coated with two control lines and five species-specific probes. After PCR products had bound to their respective complementary probe, a highly specific washing step removed any nonspecific bound DNA. Streptavidin-conjugated alkaline phosphatase was added, the samples were washed, and hybridization products were visualized by adding a substrate for alkaline phosphatase. As previously described, developed bands were categorized as follows: score 2 represented a clear band, while score 1 denoted a weak band (Eick and Pfister, 2002). According to the manufacturer the cut-off of the test is set to 10^3 - 10^4 genome equivalents.

The Pearson's correlation coefficient was used to test the relationship between probing depth and prevalence of periodontal pathogens and metabolic control.

Results

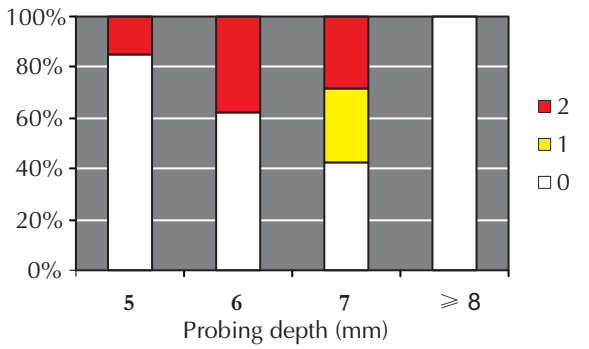
As shown in *Table 1* the highest number of periodontal pathogens was found in 5 - 7 mm deep periodontal pockets and a few at 8 mm and deeper. In 38 subgingival samples, *T. forsythia* was found most frequently (N = 20, 48%) followed by *T. denticola* (N = 13, 31%) and *P. gingivalis* (N = 11, 26%) and *P. intermedia* (N = 4, 9%). *A. actinomycetemcomitans* was found only in three samples (7%).

Figure 1. Estimated quantity of A. actinomycetemcomitans in 38 plaque samples taken from sites with different probing depths. The correlation between the bacteria and probing depth was not significant (r = 0.047, NS)



0 = no detection, 1 = weak band: ± (10^3) or + (10^3) and 2 = clear band + (10^4) or more genome equivalents.

Figure 2. Estimated quantity of P. gingivalis genome equivalents in 38 plaque samples taken from sites with different probing depth. The correlation between the bacteria and probing depth was not significant (r = 0.089, NS)

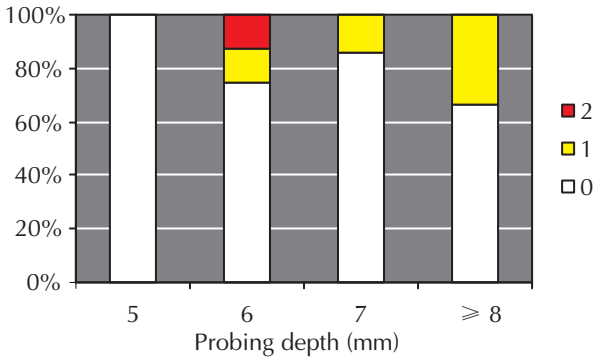


0 = no detection, 1 = weak band: ± (10^3) or + (10^3) and 2 = clear band + (10^4) or more genome equivalents.

In *Figure 1* the estimated quantity of *A. actinomycetemcomitans* genome equivalents in 38 plaque samples is shown. In plaque samples from pockets with a probing depth (PD) of 5 mm there was a clear band in 10%, and at 6 mm probing depths there was a weak band in 15%. There was no detection of *A. actinomycetemcomitans* at probing depths of 7 - 8 mm and deeper.

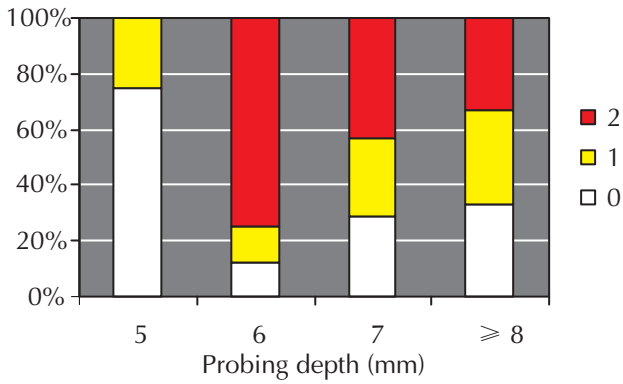
In *Figure 2* the percentage of *P. gingivalis* genome equivalents in 38 plaque samples is shown. In plaque samples from probing depths of 5 mm there was a clear band in 15% and at 6 mm there was a clear band in 38%. At 7 mm there was a clear band in 25% and a weak band in 28%. There was no detection of *P. gingivalis* at probing depths of 8 mm and more.

Figure 3. Estimated quantity of P. intermedia genome equivalents in 38 plaque samples taken from sites with different probing depths. The correlation between the bacteria and probing depth was significant (r = 0.394, p = 0.001)



0 = no detection, 1 = weak band: ± (10^3) or + (10^3) and 2 = clear band + (10^4) or more genome equivalents.

Figure 4. Estimated quantity of T. forsythia genome equivalents in 38 plaque samples taken from sites with different probing depths. The correlation between the bacteria and probing depth was significant (r = 0.202, p = 0.0124)



0 = no detection, 1 = weak band: ± (10^3) or + (10^3) and 2 = clear band + (10^4) or more genome equivalents.

In *Figure 3* the percentage of *P. intermedia* genome equivalents in 38 plaque samples is presented. In plaque samples from a probing depth of 5 mm there was no detection, at 6 mm probing depth there was a clear band in 12% and a weak band in 13%. At 7 mm probing depth there was a weak band in 14%, and at 8 mm and more there was a weak band in 36%.

In *Figure 4* the percentage of *T. forsythia* genome equivalents in 38 plaque samples is shown. In plaque samples from probing depths of 5 mm there was a weak band in 25% and at 6 mm probing depth there was a clear band in 75% and a weak band in 12%. At 7 mm there was a clear band in 42% and a weak band in 30%, and at 8 mm and deeper there was a clear band in 42% and a weak band in 30%.

Table 1. Probing depth (PD) and number of samples positive for each pathogen

PD (mm)	N of samples	*Aa*	*Pg*	*Pi*	*Tf*	*Td*	Total
5	20	2	4	0	6	3	15
6	8	1	3	2	7	4	17
7	7	0	4	1	5	5	15
8	3	0	0	1	2	1	4
	Total	3	11	4	20	13	51

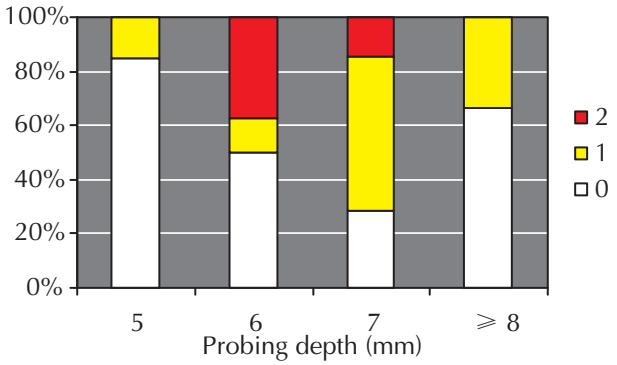
Aa, Aggregibacter actinomycetemcomitans; Pi, Prevotella intermedia; Pg, Porphyromonas gingivalis; Tf, Tannerella forsythia; Td, Treponema denticola

Table 2. The presence of periodontal pathogens and association with metabolic control

Detection of *Tf*	N of patients	HbA1c%	
+	9	7.5 ± 1.4	F-test, $p = 0.001$
-	12	6.8 ± 0.5	r = 0.23, $p = 0.015$
Detection of *Td*			
+	8	7.5 ± 1.8	F-test, $p = 0.001$
-	13	7.0 ± 0.5	r = 0.41, $p = 0.028$

Tf, Tannerella forsythia; Td, Treponema denticola

Figure 5. Estimated quantity of T. denticola genome equivalents in 38 plaque samples taken from sites with different probing depths. The correlation between the bacteria and probing depth was significant (r = 0.266, p = 0.041)



0 = no detection, 1 = weak band: ± (10^3) or + (10^3) and 2 = clear band + (10^4) or more genome equivalents.

In *Figure 5* the percentage of *T. denticola* genome equivalents in 38 plaque samples is shown. In plaque samples from a probing depth of 5 mm there was a weak band in 15% and at 6 mm probing depth there was a clear band in 38% and a weak band in 12%. At 7 mm there was a clear band in 15% and a weak band in 55%, and at 8 mm and more there was a weak band in 34%.

The evaluation of blood samples from 21 patients with type 1 diabetes showed different levels of metabolic control, according to the serum level of HbA1c. The serum level was found to be in the range of 6.2 - 9.2% (mean 7.2 ± 0.7% HbA1c).

When we evaluated the presence of five periodontal pathogens in plaque samples and metabolic control in patients we found that *T. forsythia* and *T. denticola* were significantly more frequently found in patients with higher levels of serum HbA1c (*Table 2*). The correlation coefficient value for *T. forsythia* was 0.23 ($p = 0.015$) and for *T. denticola* it was 0.41 ($p = 0.028$). No correlation was found between the presence of *P. gingivalis*, *P. intermedia*, *A. actinomycetemcomitans* and metabolic control of patients.

Discussion

The results of our study show that the presence of periodontal pathogens in subgingival plaque samples is different according to probing depth and according to the metabolic control of diabetic patients. In our study no periodontal pathogens were found in subgingival plaque samples where probing depth was 4 mm or less. The highest percentage of genome equivalents of periodontal pathogens were found at probing depths of 6 mm and 7 mm, indicating the highest inflammatory activity. In subgingival plaque samples taken from those pockets *T. forsythia* and *T. denticola* were most frequently found. Only those two periodontal pathogens correlated with poorer metabolic control. That confirms previous research where the relationship between periodontal condition and oral glucose tolerance was analyzed. They found that deep pockets (mean pocket depth > 2.0 mm) were significantly associated with impaired glucose tolerance and with diabetes as compared to shallow pockets (< 1.3 mm;

Saito *et al.*, 2004). Also, in the study by Thorstensson (1995), *P. gingivalis* was recovered more frequently from individuals with diabetes than from those without diabetes. It was recovered as often from sites with shallow pockets as from sites with deep pockets. Different results were obtained in the study by Tervonen, where they evaluated the presence of selected periodontal bacterial pathogens based on an immunoassay utilizing bacteria-specific monoclonal antibodies. Thirty-five percent of the sites harbored *P. gingivalis*, 28% *Fusobacterium nucleatum* and 21% *Eikenella corrodens*. *A. actinomycetemcomitans* and *P. intermedia* were found in less than 10% of the sites. Subjects for whom the probing depth at the sampled site was $\geqslant 4$ mm were more often found to have detectable pathogens than those with a probing depth $\leqslant 3$ mm. Diabetic factors such as duration, type and metabolic control of the disease had no statistically significant effect on the prevalence of these bacteria (Tervonen *et al.*, 1994). In that study they did not evaluate the presence of *T. forsythia* and *T. denticola.*

In contrast to the majority of previous studies where metabolic control was associated mainly with the presence of *P. gingivalis*, in our study *T. forsythia* and *T. denticola* were most frequently found to correlate with metabolic control. In some previous studies *T. forsythia* was associated with periodontal disease but not with diabetes mellitus. A predominance of *T. forsythia* was shown in a study by Aimetti (2007), where in 21 patients with chronic periodontal disease *T. forsythia* was detected in 72.22% of periodontal pockets, followed by *P. gingivalis*, *P. intermedia*, *T. denticola* and *A. actinomycetemcomitans* in 61.11%, 55.56%, 50%, and 33.33% respectively.

When analyzing the pathogenicity of *T. forsythia,* the pathogenic effect of lipopolysaccacharide (LPS) and bacterial DNA from *T. forsythia* were shown to induce production of proinflammatory cytokines by human macrophages, with the IL-8 secretion level of LPS from *T. forsythia* being about 1.5 times the effect of LPS from *P. gingivalis* (Bodet *et al.*, 2006; Sahingur *et al.*, 2010). *T. forsythia* could be internalized by macrophages and stimulate intracellular receptors. The responses of macrophages upon stimulation with *T. forsythia* are complex, involving autocrine responses to produced cytokines (Sekot *et al.*, 2010). One hypothesis proposes that periodontal infection-mediated cytokine synthesis and secretion may amplify the magnitude of the advanced glycation endproducts (AGE)-mediated cytokine response and vice versa.

The relationship between diabetes and periodontal disease becomes a two-way relationship (Grossi and Genco, 1998). Both diabetes and periodontal disease can stimulate the chronic release of proinflammatory cytokines that have a deleterious effect on periodontal tissues (Naguib *et al.*, 2004). The chronic systemic increase of proinflammatory cytokines caused by periodontal disease may even predispose individuals to the development of type 2 diabetes (Iacopino, 2001). A model was presented by Pucher (2004), in which periodontal pathogens may cause an increase in proinflammatory cytokines that mediate an increase in insulin resistance, resulting in an increase in blood glucose. Following periodontal therapy, this process may be reversed.

Another effect of *T. forsythia* is that not only does it promote periodontal disease and affect the metabolic control of diabetic patients, but it also relates to the body mass index (BMI), which affects diabetes. Certain cytokines cause cells to be resistant to the effects of insulin, leading to diabetes and heart disease. Macrophages in fat tissue produce cytokines that prevent cells from appropriately responding to the presence of insulin (Wentworth *et al.*, 2010). In a study by Haffajee and Socransky (2009) there was a strong association between BMI and periodontal status in the subjects younger than 46.8 years. The increased proportions of *T. forsythia* were due to a significant increase in periodontal pockets < 4 and 4–6 mm in obese individuals. Other species of the red complex, *P. gingivalis* and *T. denticola*, as well as the other test species, did not demonstrate this relationship (Haffajee and Socransky, 2009).

T. forsythia has been associated with BMI and gestational diabetes mellitus. When cases were compared with healthy control individuals, higher pre-pregnancy body mass index ($p = 0.004$), vaginal levels of *T. forsythia* ($p = 0.01$), serum C-reactive protein ($p = 0.01$), and prior gestational diabetes mellitus ($p = 0.006$) emerged as risk factors, even though the clinical periodontal disease failed to reach statistical significance (Dasanayake *et al.*, 2008).

The pathogenicity of *T. forsythia* and its impact on periodontal disease and diabetes was proposed previously, and was confirmed with the results of our study by the presence of *T. forsythia* in deep periodontal pockets and its influence on metabolic control.

Conclusions

Our research has shown the influence of periodontal pathogens on the metabolic control of diabetic patients. The presence of five different periodontal pathogens was tested in plaque samples from pockets with different probing depths. *T. forsythia* and *T. denticola* were the most frequently found bacteria in subgingival plaque samples in our study, and only the presence of these two periodontal pathogens correlated with poorer metabolic control in type 1 diabetes patients. These findings need to be confirmed in larger studies with different demographics to contribute to diagnostic sensitivity and treatment needs of periodontal disease and diabetes mellitus.

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