Clin Oral Invest (2008) 12:73–81
DOI 10.1007/s00784-007-0147-7

ORIGINAL ARTICLE

Detection of *Treponema denticola* in saliva obtained from patients with various periodontal conditions

María Cecilia Martínez-Pabón · Diana Patricia Restrepo-Ospina · Diana María Isaza-Guzmán · Luz Marina Orozco-Rojas · Sergio Iván Tobón-Arroyave

Received: 30 April 2007 / Accepted: 16 August 2007 / Published online: 7 September 2007

Abstract The aim of the study was to determine the prevalence of *Treponema denticola* in saliva of periodontally diseased and healthy patients and its relationship with the periodontal status. A 16S rRNA-based polymerase chain reaction detection method was used to determine the prevalence of *T. denticola* in whole saliva samples from patients with chronic periodontitis (CP, n=37), aggressive periodontitis (AgP, n=24), and healthy subjects (n=28). The periodontal status of each subject was assessed by criteria based on probing depth, clinical attachment loss, and extent of periodontal breakdown. Risk factors were assessed individually and adjusted for confounding using a binary logistic regression model. The results showed that the prevalence of *T. denticola* in CP patients was significantly higher than those in healthy and AgP subjects (P<0.05). Odds ratio analysis revealed a positive association for CP group/*T. denticola*-positive and smoking/*T. denticola*-positive subjects. Furthermore, all clinical measurements were significantly greater (P<0.05) for *T. denticola*-positive subjects compared to *T. denticola*-negative subjects. After binary logistic regression analysis, both *T. denticola* and smoking were independently and strongly associated with development of CP. It was concluded that when used in conjunction with an optimized clinical examination protocol, this assay may offer a rapid, useful, and cost-effective tool for monitoring the presence of *T. denticola* in noninvasive clinical samples from both healthy and diseased patients and correlating it with the amount and extent of periodontal breakdown.

Keywords Aggressive periodontitis · Chronic periodontitis · 16S rRNA · PCR · Saliva · *Treponema denticola*

M. C. Martínez-Pabón · D. P. Restrepo-Ospina · D. M. Isaza-Guzmán · L. M. Orozco-Rojas · S. I. Tobón-Arroyave (✉)
POPCAD Research Group, Laboratory of Immunodetection and Bioanalysis, Faculty of Dentistry, University of Antioquia, Calle 64 No 52-59, P.O. Box: 1226, Medellín, Colombia
e-mail: labinbio@odontologia.udea.edu.co

Introduction

Periodontitis is a disease of multifactorial nature with pathogenesis related to several risk factors, including bacterial pathogens, host responses, and possibly certain genetics traits [17]. It is characterized by the irreversible destruction of collagen fibers and other matrix constituents of the gingiva, periodontal ligament, and alveolar bone around the teeth in conjunction with the formation of periodontal pockets due to the apical migration of the junctional epithelium [21]. The prevalence of periodontitis in humans is approximately 30%, 10–15% of whom have a severe form of the disease [20].

Spirochetes of genus *Treponema* are a major component of the bacterial flora of the oral cavity [4, 23]. Of these, *Treponema denticola* has been predominantly associated with the incidence and severity of human periodontal disease [15]. This aerotolerant, anaerobic spirochete [23] dwells in a complex and diverse microbial community within the oral cavity and as such is highly specialized to survive within this milieu [32], being especially prevalent in the anaerobic gingival crevice [23].

In the periodontal pocket, *T. denticola* has been found close to the junctional epithelium and has shown to adhere to epithelial cells and fibroblasts as well as to matrix proteins [37]. It is also able to adhere to other bacteria [44],

to collagen-binding proteins [41], and to collagen used as a barrier for guided bone regeneration [31]. Of special interest are the bimodal and nutritional interactions between *T. denticola* and other periodontal bacteria, especially *Porphyromonas gingivalis* [44]. The cells in contact with *T. denticola* show cytopathic changes, such as membrane blebbing, vacuolization, inhibition of motility, and loss of epithelial cell contacts. Migrating and proliferating cells are more sensitive to the action of *T. denticola* than mature cells of epithelial layers that appear to develop a limited resistance to the organism. In contrast, the motile epithelial cells have the ability to internalize and destroy the bacteria [38]. The presence of several putative virulence factors such as an outer membrane-associated chymotrypsin-like enzyme, phospholipase C activity, a pore-forming outer sheath protein, and cytotoxic proteoglycan in these microorganisms [18, 38], together with their high motility [24], may contribute to the invasive capability of treponemes. Moreover, due to oral treponemes having adherent properties to epithelial cells and coaggregation abilities to important biofilm-bridging organisms [44], the mechanical dispersal of dental plaque containing treponemes may lead to the colonization of new intraoral habitats after periodontal therapy [5].

As it is difficult to isolate and identify spirochetes from clinical plaque samples, currently, the 16S rRNA-based polymerase chain reaction (PCR) method continues to be one of the most sensitive, rapid, and cost-effective methods for determining the prevalence of such microorganisms [27, 28]. In addition, as saliva is the probable vehicle for transmission of oral bacteria, microorganisms found in saliva may derive from various oral surfaces, including gingival crevices and pockets [10]. Given that periodontal pocket bacteria are continuously washed into saliva by gingival crevicular fluid, a whole saliva sample may offer a rapid and easy alternative to individual pocket samples that can be performed in any clinical setting for determining subgingival bacterial presence when a sensitive microbiological detection technique such as PCR is employed [28, 36, 39].

The aim of this study was to determine the prevalence of *T. denticola* by 16S rRNA-based PCR technique in whole saliva of periodontally diseased and healthy patients and its relationship with the periodontal status.

Materials and methods

Patient selection and inclusion/exclusion criteria

A total of 89 patients attending the Graduate Periodontics Clinic at the University of Antioquia as well as patients from the private practices of periodontists were recruited for the study. All participants provided written informed consent before their enrolment into the study. The study conformed to the ethical guidelines of the Helsinki Declaration and was evaluated and approved by the Institutional Research Ethics Board (CIFO).

Participants were privately interviewed to obtain medical and demographic information and were given a clinical screening for oral pathology and a periodontal examination. The information that was gathered included the subject's gender, age, and tobacco use. A subject was considered a nonsmoker if he or she had never smoked or had stopped smoking more than 5 years previous to the date of examination.

All clinical periodontal measurements were performed by a single calibrated investigator (DR). The clinical parameters recorded, including probing depth (PD) and clinical attachment loss (CAL), were measured at six surfaces of all teeth (midbuccally, midlingually, and proximally both buccally and lingually) to the nearest millimeter using a straight periodontal probe (PCP UNC 15, Hu-Friedy, Chicago, IL, USA). PD was defined as the distance from the coronal-most margin of the free gingival to the most apical penetration of periodontal probe. As a reference for CAL measurement, the cemento–enamel junction (CEJ) or the apical border of the restoration, if the CEJ was not visible, was used. Following previously defined criteria [1, 17], these measurements were used to calculate the extent of periodontitis based on the percentage of tooth sites having PD ≥4 mm along with CAL ≥2 mm. All calculations were based on data derived from the full-mouth examination.

Subjects for this study comprised 37 chronic periodontitis (CP) and 24 aggressive periodontitis (AgP) patients. These patients were classified as follows: CP, subjects having a minimum of 20 remaining teeth, with periodontal disease as evidenced by at least four tooth sites with PD ≥4 mm, CAL ≥3 mm, and radiographic evidence of extensive bone loss [14]; AgP, subjects with a pattern of severe destruction, with attachment loss of at least 5 mm on eight or more teeth, at least three of which were not first molars or incisors (generalized) or first molar/incisor presentation with interproximal attachment loss on at least two permanent teeth (localized), one of which was a first molar [11]. As healthy controls, 28 subjects with no evidence of pocket depth or attachment loss measurements >3 mm and no gingival inflammation were also included.

Exclusion criteria included pregnancy; any systemic condition that could affect the host's periodontal status (e.g., diabetes mellitus, immunological disorders, AIDS) or that would require antibiotics for monitoring or treatment procedures (e.g., heart conditions, joint replacements); use of antibiotics and/or anti-inflammatory drugs within the last 3 months; and professional cleaning or periodontal treatment within the last 6 months.

Saliva collection and processing

About 10 ml of unstimulated whole saliva was collected from each subject into a 50-ml sterile plastic centrifuge tube (Greiner Bio-one®, Frickenhausen, Germany) before breakfast intake and any dental hygiene procedure. No antiseptic mouth rinse was used before collection. Samples were processed at the same day, as reported by Umeda et al. [39]. Briefly, the tubes were vigorously dispersed using a vortex (IKA® Vibrofix, Staufen, Germany) for 10 s. Immediately, 0.5 ml of whole saliva was diluted 1:2 with sterile distilled water and collected by centrifugation at 9,800 rpm for 5 min in a microcentrifuge (Biofuge *fresco*/Heraeus®, Kendro Laboratory Products, Langenselbold, Germany). The resulting pellet was washed four more times with sterile water and then reconstituted with 0.5 ml water. Washed samples were kept at −70°C until processing.

DNA extraction

The saliva specimens were prepared for PCR according to the procedure of Sánchez-Jiménez and Cardona-Castro [29]. Briefly, 200 μl of washed saliva were centrifuged at 10,000 rpm for 5 min. The pellet was mixed with 200 μl of 10 mM Tris/HCl, pH 8, and 1 mM ethylenediamine tetraacetic acid (EDTA) and centrifuged at 12,000 rpm for 6 min. This procedure was performed twice. Then, the pellet was mixed with 200 μl distilled water and centrifuged for 1 min at 12,000 rpm, and 30 μl distilled water was added to the pellet followed by incubation at 100°C for 20 min. Supernatant was used for PCR analysis.

PCR detection

T. denticola PCR detection was performed using primers and methods previously described [2] with some modifications. Briefly, 10 μl of DNA sample was added to 50 μl of PCR mixture containing, 5 μl 10× PCR buffer (Promega, Madison, WI, USA), 0.2 mM of each of deoxyribonucleotides (Promega), 1.0 μM of each primer (IDT®, Coralville, IA,USA), 2 mM $MgCl_2$ (Promega), and 1.25 U *Taq* DNA polymerase (Promega). A pair of primers (5′-TAA TAC CGA ATG TGC TCA TTT ACA T-3′ and 5′-TCA AAG AAG CAT TCC CTC TTC TTC TTA-3′) targeted 16S rRNA genes, amplifying a fragment of 316 bp length (base positions 193–508). PCR amplification was carried out in a 96-well PTC-100 thermal cycler (MJ Research, Waltham, MA, USA). Samples were initially denatured at 95°C for 2 min, followed by 36 cycles of 95°C for 30 s, 60°C for 1 min, 72°C for 1 min, and a final extension step of 72°C for 2 min. The specificity of the primer pair has been thoroughly proven in other studies [2, 28, 32, 34].

PCR products were analyzed by 1.5% agarose gel electrophoresis, stained with 0.5 μg/ml ethidium bromide, visualized, and digitized using a BioRad Gel Doc 2000 System (BioRad Laboratories, Athens, OH, USA). As molecular size marker, 1 kb DNA ladder (Promega) was used. Positive and negative controls were included in each PCR assay. *T. denticola* ATCC 35405 genomic DNA was used as a positive control, which was kindly purchased by Professor SJ Norris (University of Texas at Houston, USA). For negative control, DNA sample was replaced by sterile water. The sensitivity of the PCR assay was determined by performing PCR in reactions containing serial dilutions of *T. denticola* ATCC 35405 purified genomic DNA. To assess the possible inhibition of PCR reaction by saliva components, a test using a pool of saliva from 15 patients randomly selected of the study population was done. Subsequently, 10 μl of each washed saliva sample and 10 ng of pure *T. denticola* genomic DNA were mixed, and an aliquot of 5 μl was used for PCR reaction.

Statistical analysis

Data collected were analyzed using Statistical Package for the Social Sciences (SPSS) 14.0® (SPSS, Chicago, IL, USA). All parameters were tested for normal distribution using the Shapiro–Wilk test for small sample size. Because the results for each group did not follow a normal distribution, the variables were analyzed using nonparametric methods. All variables were summarized according to the clinical groups for all subjects. The Pearson's chi-square test (χ^2) was used to assess differences in gender and tobacco use between the clinical groups. To determine the differences in age, PD, and CAL between groups, the Kruskal–Wallis *H* test followed by Mann–Whitney's post-hoc comparisons were performed. The latter was also used to determine differences between CP and AgP groups regarding the extent of the disease.

Detection frequencies of *T. denticola* were calculated from the PCR results of saliva samples and are given as the percentage of *T. denticola*-positive subjects. The odds ratio (OR) calculation with 95% confidence interval was applied to examine the relationship of the percentage of *T. denticola*-positive subjects with the clinical groups and tobacco use. The significance of the association was determined by the Pearson's chi-square test. A positive association existed when the OR was greater than 2 and the confidence range did not include 1.0. Differences in clinical parameters between *T. denticola*-positive and -negative subjects were assessed using the Mann–Whitney *U* test.

Finally, to test the strength and independence of the association of each significant risk factor with development of periodontitis, a binary logistic regression model was used. Risk factors with a level of significance defined as

$P<0.20$ in univariate analysis were adjusted in a multivariate explanatory analysis. In this analysis, each candidate risk factor was evaluated after adjusting for other candidate risk factor and for potential confounding variables. Significance for all tests was established at a P value <0.05. Due to descriptive nature and explorative character of this study, P values were not adjusted, despite multiple comparisons.

Results

Demographic and clinical characteristics of the study groups and controls are displayed in Table 1. Although women comprised about 64% of the patients studied, there were no significant differences between men and women in the study groups compared with healthy controls ($P>0.05$, χ^2). The age of the subjects ranged between 18 and 72 years, and a statistically significant difference was observed in the median ages between the three study groups ($P<0.001$, Kruskal–Wallis test). While the age of the CP group was significantly greater than AgP and healthy subjects ($P<0.001$, Mann–Whitney U test), there was no significant difference between healthy and AgP patients ($P=0.173$). Overall, the patients in the CP group were significantly more smokers ($P<0.001$, χ^2). As also shown in Table 1, median values of PD and CAL in healthy subjects were significantly lower than those of periodontitis patients ($P<0.001$, Mann–Whitney U test). However, no significant difference ($P>0.05$) was evident for these parameters or for extent of periodontitis between the two periodontitis groups.

With regard to PCR detection of *T. denticola*, no bands of the predicted size were produced with nontarget DNA. The sensitivity of the PCR detection limit for *T. denticola* genomic DNA was established at 10^{-5} ng (Fig. 1), and PCR inhibition assay demonstrated that saliva prepared as described did not interfere with amplification of *T. denticola* DNA (Fig. 2). The results from representative samples in which the presence of *T. denticola* was detected are shown in Fig. 3. The primers amplified a band of the predicted size (316 bp), and no amplification of different sized bands was observed. Positive control yielded the expected size amplicon, and there were no bands in the negative control. While detection frequency of *T. denticola* in saliva samples from CP patients was significantly higher than those in healthy ($P=0.030$, χ^2) and AgP subjects ($P=0.039$), the differences between AgP and healthy groups were not statistically significant ($P=0.335$, Table 2). In addition, detection frequency of *T. denticola* was significantly greater in smokers compared to nonsmoker subjects ($P=0.034$, Table 3). OR analysis revealed a positive association for CP group/*T. denticola*-positive and smoking/*T. denticola*-positive subject combinations (Tables 2 and 3).

After adjustment for confounding in the multivariable analysis, the risk factors associated with development of CP were *T. denticola*-positive subjects and smoking habit. These two variables were fitted into a binary logistic regression model. This final model with adjusted ORs is presented in Table 4.

The comparison of clinical measurements between *T. denticola*-positive and negative subjects are summarized in Fig. 4. Overall, median values of PD, CAL, and extent of

Table 1 Demographic and clinical characteristics of study subjects

Characteristics		Clinical groups			P value
		Healthy controls ($n=28$)	Chronic periodontitis ($n=37$)	Aggressive periodontitis ($n=24$)	
Gender	Men	10	24	9	0.982[a]
	Women	18	13	15	
Age (years)[b]		30.5 (24.0–72.0)	45.0 (29.0–66.0)[c]	31.5 (18.0–41.0)	<0.001[d]
Tobacco use	Nonsmoking	26	23	23	0.001[a]
	Smoking	2	14[c]	1	
Mean PD[e] (mm)[b]		1.68 (1.24–2.49)	3.30 (1.43–6.66)[c]	3.52 (2.39–6.31)[c]	<0.001[d]
Mean CAL[e] (mm)[b]		0.71 (0.40–3.60)	3.40 (1.33–7.70)[c]	3.54 (0.39–7.41)[c]	<0.001[d]
Extent[f] of periodontitis[c]		0	35.70 (3.62–75.00)	36.53 (8.00–80.00)	0.808[g]

[a] Two-sided χ^2 for independence
[b] The values are given as median (range)
[c] Statistically significant differences as compared with healthy controls
[d] Two-sided Kruskal–Wallis H test
[e] Data based on measurements obtained from the entire dentition
[f] Percentage of periodontal pockets ≥4 mm deep and attachment loss ≥2 mm
[g] Two-sided Mann–Whitney U test

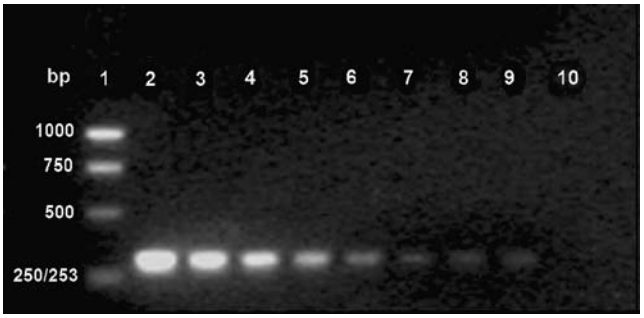

Fig. 1 Electrophoresis results of PCR detection limit of *T. denticola* ATCC 35405 genomic DNA. *Lane 1* 1 kb ladder; *lanes 2–10* serial dilutions from 10^{2} to 10^{-6} ng of pure *T. denticola* genomic DNA

periodontal involvement were significantly greater for *T. denticola*-positive subjects compared to *T. denticola*-negative subjects ($P<0.05$, Mann–Whitney U test).

Discussion

Bacterial infections may cause disease manifestations through multiple mechanisms, including, but not limited to, direct effects of bacterial products, effects of the host immune response to the organism, and the persistent actions of the host immune response after clearance of the organism [13]. Although there is little question that the signs and/or symptoms of periodontal disease result from the presence of the microorganisms, the cause of periodontal breakdown in patients with AgP and CP is more controversial. As saliva is known to reflect the total bacterial profile of an individual and provide a representative view of the bacterial population in the entire oral cavity [36], the present study was conducted to evaluate the prevalence of *T. denticola* in saliva of patients with CP/AgP and periodontally healthy subjects and its relationship with the periodontal status.

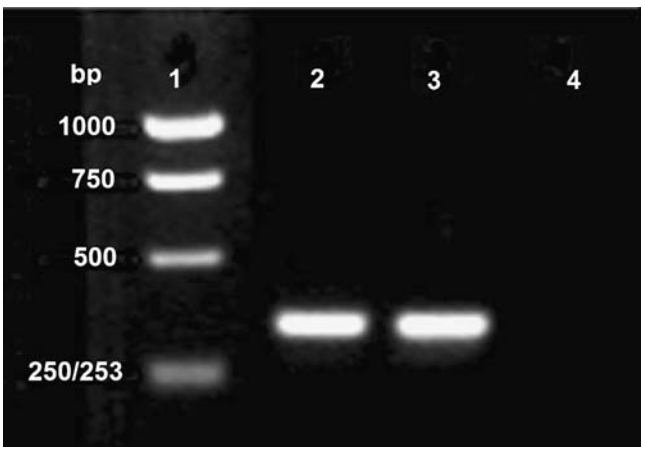

Fig. 2 Electrophoresis results of PCR inhibition assay. *Lane 1* 1 kb ladder; *lane 2* pool of saliva samples; *lane 3* positive control (10 ng of *T. denticola* DNA as template); *lane 4* negative control (sterile water)

Demographic characteristics of study subjects showed statistically significant differences in age and tobacco use according to parameters agreed for AgP and CP [11, 14]. A full-mouth examination was used to evaluate some clinical parameters associated with prevalence and severity of periodontal disease and to allow direct comparisons among the clinical groups studied, as it could give suggestive information about contrasting patterns of disease among different study groups. Thus, in this study, the clinical analysis indicated a generalized form of periodontal disease in both CP and AgP groups (>30% of sites involved) [1], with amounts of PD and CAL statistically similar between them.

Previous studies had indicated that periodontopathic bacteria are more consistently detected in saliva than in subgingival plaque samples [28, 39]. The levels of target bacteria may vary considerably from pocket to pocket, and a pooled sample from sites of periodontitis may occasionally have missing organisms. It may also be that whole saliva simply contains higher concentrations of target bacteria than subgingival plaque samples [28]. It has been reported previously [39] that ≥10 cells/5 μl were required for detection of *T. denticola* in whole, untreated saliva, most likely due to an inhibitory effect of saliva. In this study, saliva specimens were centrifuged and washed five times before PCR testing, thereby concentrating any bacteria present and removing potential inhibitors [28]. The limit of detection in the PCR reaction was 10^{-5} ng, corresponding to three *T. denticola* genome copies/200 μl saliva (15 cells/ml saliva) as calculated by inference of published genomic data for *T. denticola* [32]. Although this increased PCR sensitivity may detect levels of *T. denticola* too low to be clinically significant, it could indicate a risk factor for periodontitis in healthy subjects.

Several studies using subgingival plaque samples have shown that *T. denticola* is more frequently detected in periodontally diseased subjects than in healthy subjects [26, 33, 35]. Nevertheless, whereas it has been demonstrated that saliva is equal to or better than subgingival plaque for detecting and quantifying periodontopathic bacteria in the

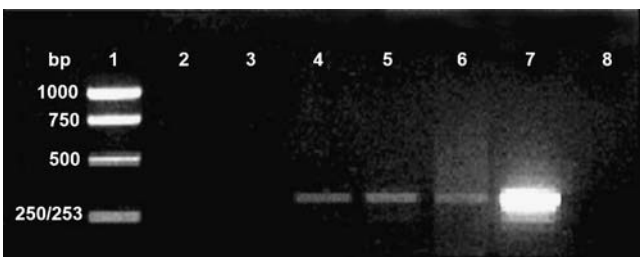

Fig. 3 Electrophoresis results of PCR amplification of *T. denticola* in saliva samples. A single DNA band of the predicted size (316 bp) was obtained by PCR using specific primer pair against the target organism. *Lane 1* 1 kb ladder; *lanes 2–6* saliva samples with different periodontal diagnosis; *lane 7* positive control (10 ng of *T. denticola* DNA as template); *lane 8*, negative control (sterile water)

Table 2 Salivary detection frequencies and odds ratios of association among *T. denticola* and clinical groups

Clinical groups	*T. denticola*-positive subjects *n* (%)	Odds ratio	*P* value[a]
Healthy controls (*n*=28)[b]	5 (17.9)	1	
Chronic periodontitis (*n*=37)	16 (43.2)	3.505[c] (1.09~11.24)	0.030
Aggressive periodontitis (*n*=24)	7 (29.2)	1.894[c] (0.51~7.00)	0.335

[a] Two-sided χ^2 for independence
[b] Reference group
[c] Odds ratio (95% confidence interval)

oral cavity [28, 36, 39], only partial information is available regarding detection of *T. denticola* in saliva samples and its association with different periodontal diagnosis [34].

The result of the OR statistic in the present study showed a positive association between CP subjects and *T. denticola*. This finding concurs with previous reports where *T. denticola* has been specifically implicated in CP [6, 30]. More recently, it has been also suggested that *T. denticola* may be one of the principal bacteria observed in AgP [34]. In this study, a significantly higher detection frequency of *T. denticola* was obtained in saliva samples from CP patients (43.2%) in comparison with those from AgP and healthy subjects (29.2 and 17.9%, respectively). The differences in *T. denticola* detection between AgP and healthy subjects were not statistically significant. These results suggest that the mechanism by which the severe and rapid periodontal breakdown occurs in AgP might be independent of the presence of *T. denticola* and it is likely to be by the involvement of other bacteria with higher pathogenicity, such as *Tanerella forsythensis* and *P. gingivalis*, as noted by others [35]. Furthermore, several factors that affect the immune response of the host, among them neutrophil function abnormalities [22] or excessive production of inflammatory cytokine/prostaglandin [19], are likely to indirectly modulate the increased periodontal destruction observed in AgP.

On the other hand, the current investigation is in agreement with previous studies that found detectable levels of putative periodontal pathogens on the soft tissues and in the saliva of periodontally healthy subjects [16, 34, 36, 43]. Given that a pathogen may need to be in numbers sufficient to exceed a particular threshold to initiate disease, *T. denticola* may be found in healthy subjects who may be referred to as being in a carrier state [43]. Consequently, although the concentration of periodontal pathogens in subgingival sites is the critical factor that will determine whether disease will occur or progress, it is quite probable that saliva might act as reservoir so that *T. denticola* colonizes these sites contributing to the onset of periodontitis when other factors such as high levels of one or more specific pathogens, low levels of beneficial species, and environmental/genetic features associated with a susceptible host are also present [21].

The results of this study revealed that the patients in the CP group were significantly more smokers than AgP and healthy subjects (Table 1), and that smokers were significantly more likely to harbor *T. denticola* in their saliva (OR 3.138; Table 3). Although after binary logistic regression analysis both *T. denticola* and smoking were independently and strongly associated with development of CP, from the data presented, it seems that the presence of *T. denticola* in saliva might be more related to the smoking habit than to the severity of periodontal disease. Although various studies have reported that smoking and/or periodontal disease are associated with significant changes in the colonization patterns of certain species on soft tissues or in saliva [3, 42], limited information is available with regard to the changes in the prevalence of *T. denticola* in smokers and nonsmokers. The current investigation is in agreement with previous studies that found a significantly

Table 3 Salivary detection frequencies and odds ratios of association among *T. denticola* and tobacco use

Tobacco use	*T. denticola*-positive subjects *n* (%)	Odds ratio	*P* value[a]
Nonsmoking (*n*=72)[b]	19 (26.4)	1	
Smoking (*n*=17)	9 (52.9)	3.138[c] (1.06~9.31)	0.034

[a] Two-sided χ^2 for independence
[b] Reference group
[c] Odds ratio (95% confidence interval)

Table 4 Binary logistic regression analysis: significant risk factors for development of chronic periodontitis

Risk factors	Unadjusted OR (95% CI)[a]	Adjusted OR (95% CI)[a]	*P* value[b]
T. denticola-positive subjects	3.505 (1.09~11.24)	3.695 (1.08~12.65)	0.037
Smoking subjects	7.913 (1.62~38.58)	8.274 (1.64~41.81)	0.011

[a] Odds ratio (95% confidence interval)
[b] Wald test

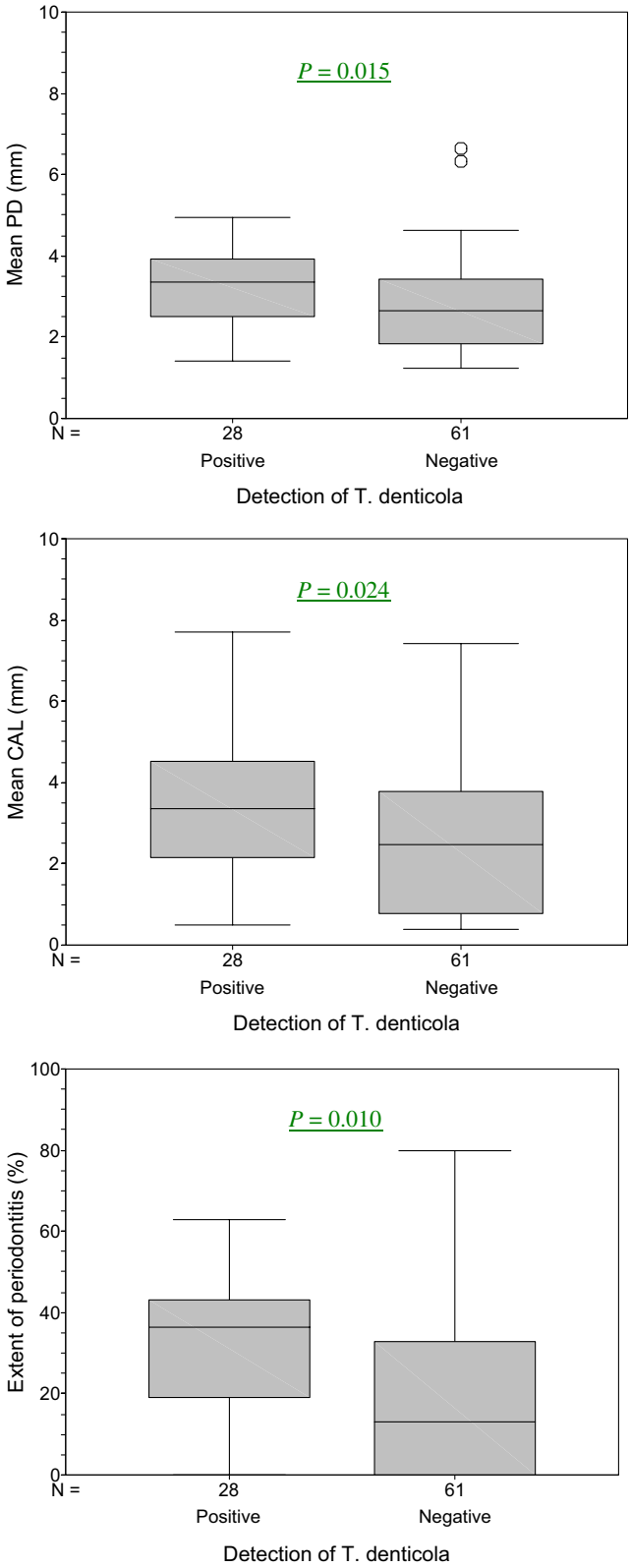

Fig. 4 Quantitative comparison of clinical measurements obtained from *T. denticola*-positive and negative subjects as detected in saliva samples. The *box* represents the first and third quartiles (*rectangular boxes*); the *line within the box* is the median; *vertical bars* show the 95% confidence interval. *Little circles* above these levels represent atypical values that were plotted separately. All differences were statistically significant at level $P<0.05$

higher risk of smokers for harboring *T. denticola* in oral cavity than nonsmokers [7, 40]. Other authors [16] have also reported higher proportions of *T. denticola* colonizing soft tissues and saliva in smoking subjects, but these elevations were not statistically different. Although the reason for differences in the composition of the microbiota between smokers and nonsmokers is not completely understood, a number of possible explanations exist. It is well known that smoking exerts profound effects on the vascular and immune systems [8, 25]. Another possible explanation is that the dramatic increase in anaerobiosis, resulting of decreased oxygen tension which occurs in the oral cavities of smokers, promotes the growth of anaerobic bacterial species [9]. The results of the present study argue against the opinion that microbiota of various sites appears to be minimally affected by the subject's smoking habits or by the presence or absence of periodontal disease [12]. A partial explanation for the disagreement could be attributed to the sensitivity and the specificity in the detection techniques used as well as to the differences to the sampling method and study population.

Regarding clinical parameters, this study demonstrated that the amount of periodontal breakdown appeared to be influenced by the presence of *T. denticola*, as statistically significant elevations were seen in the median values of PD, CAL, and extent of periodontitis in *T. denticola*-positive compared to -negative subjects. These findings are consistent with those described by others [33, 34] whose using different detection techniques observed a positive association between presence of *T. denticola* and periodontal tissue destruction.

Two limitations were associated with the present study. The small sample size in association with the variability of patterns of disease among different study groups might have influenced the results, as the clinical measurements of these subjects might not represent critical-size defects to evaluate the association between periodontal status, smoking habit, and presence of *T. denticola* in unstimulated whole saliva. Thus, based on the data presented herein, it is not possible to entirely reject the hypothesis that there is an increased frequency of *T. denticola* in AgP. An analysis with more patients would have greater statistical power and precision.

In summary, the findings, when considered within the limitations of this study, reemphasize the importance of whole saliva as sampling method in terms of microbiological purposes in periodontal disease. When used in conjunction with an optimized clinical examination protocol, both the sampling method and the assay may offer a rapid, useful, and cost-effective tool for monitoring the presence of *T. denticola* in noninvasive clinical samples from both healthy and diseased patients and correlating it with the amount and extent of periodontal breakdown.

Acknowledgments This research was supported by the Research Development Committee of the University of Antioquia (CODI-Code 8700-8392). The authors thank Professor Steven J. Norris (University of Texas at Houston, USA) for providing *T. denticola* ATCC 35405 purified genomic DNA. Additional thanks go to Professor Carlos Muskus (University of Antioquia) for assistance in PCR detection techniques.

References

1. Armitage GC (1999) Development of a classification system for periodontal diseases and conditions. Ann Periodontol 4:1–6
2. Ashimoto A, Chen C, Bakker L, Slots J (1996) Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions. Oral Microbiol Immunol 11:266–273
3. Colman G, Beightom D, Chalk AJ, Wake S (1976) Cigarette smoking and the microbial flora of the mouth. Aust Dent J 21:111–118
4. Correia FF, Plummer AR, Paster BJ, Dewhirst FE (2004) Genome size of human oral Treponema species by pulsed-field gel electrophoresis. Oral Microbiol Immunol 19:129–131
5. Ehmke B, Beikler T, Riep B, Flemmig T, Göbel U, Moter A (2004) Intraoral dissemination of treponemes after periodontal therapy. Clin Oral Investig 8:219–225
6. Haffajee AD, Gugini MA, Tanner A, Pollack RP, Smith C, Kent RL Jr, Soncransky SS (1998) Subgingival microbiota in healthy, well-maintained elder and periodontitis subjects. J Clin Periodontol 25:346–353
7. Haffajee AD, Socransky SS (2001) Relationship of cigarette smoking to the subgingival microbiota. J Clin Periodontol 28:377–388
8. Johnson GK, Slach NA (2001) Impact of tobacco use on periodontal status. J Dent Educ 65:313–321
9. Kenney EB, Saxe SR, Bowles RD (1975) The effect of cigarette smoking on anaerobiosis in the oral cavity. J Periodontol 46:82–85
10. Könönen E, Jousimies-Somer H, Asikainen S (1994) The most frequently isolated gram-negative anaerobes in saliva and subgingival sample taken from young women. Oral Microbiol Immunol 9:126–128
11. Lang N, Bartold PM, Cullinan M, Jeffcoat M, Mombelli A, Murakami S, Page R, Papapanou P, Tonetti M, Van Dike T (1999) Consensus report: aggressive periodontitis. Ann Periodontol 4:53
12. Lie MA, van der Weijden GA, Timmerman MF, Loos BG, van Steenbergen TJM, van der Velden U (1998) Oral microbiota in smokers and non-smokers in natural and experimentally-induced gingivitis. J Clin Periodontol 25:677–686
13. Lin B, Noring R, Steere AC, Klempner MS, Hu LT (2000) Soluble CD14 levels in the serum, synovial fluid, and cerebrospinal fluid of patients with various stages of Lyme disease. J Infect Dis 181: 1185–1188
14. Lindhe J, Ranney R, Lamster I, Charles A, Chung C-P, Flemmig T, Kinane D, Litsgarten M, Löe H, Schoor R, Seymour G, Somerman M (1999) Consensus report: chronic periodontitis. Ann Periodontol 4:38
15. Loesche WJ, Grossman NS (2001) Periodontal disease as a specific, albeit chronic, infection: diagnosis and treatment. Clin Microbiol Rev 14:727–752
16. Mager DL, Haffajee AD, Socransky SS (2003) Effects of periodontitis and smoking on the microbiota of oral mucous membranes and saliva in systemically healthy subjects. J Clin Periodontol 30:1031–1037
17. Michalowicz BS, Diehl SR, Gunsolley JC, Sparks BS, Brooks CN, Koertge TE, Califano JV, Burmeister JA, Schenkein HA (2000) Evidence of a substantial genetic basis for risk of adult periodontitis. J Periodontol 71:1699–1707
18. Mikx FH (1997) Environmental effects on the growth and proteolysis of *Treponema denticola* ATCC 33520. Oral Microbiol Immunol 12:249–253
19. Offenbacher S (1996) Periodontal diseases: pathogenesis. Ann Periodontol 1:821–878
20. Oliver RC, Brown LJ, Loe H (1998) Periodontal diseases in the United States population. J Periodontol 69:269–278
21. Page R, Kornman KS (1997) The pathogenesis of periodontitis. Periodontology 2000 14:9–11
22. Page RC, Vandesteen GE, Eversole JL, Williams BL, Dixon IL, Altman LC (1985) Clinical and laboratory studies of a family with a high prevalence of juvenile periodontitis. J Periodontol 56: 602–610
23. Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, Sahasrabudhe A, Dewhrist FE (2001) Bacterial diversity in human subgingival plaque. J Bacteriol 183:3770–3783
24. Pietrantonio F, Noble PB, Amsel R, Chan ECS (1988) Locomotory characteristics of *Treponema denticola*. Can J Microbiol 34: 748–752
25. Rawlinson A, Grummitt JM, Walsh TF, Douglas CWI (2003) Interleukin 1 and receptor antagonist levels in gingival crevicular fluid in heavy smokers versus non-smokers. J Clin Periodontol 30:42–48
26. Riviere GR, Smith KS, Carranza N Jr, Tzagaroulaki E, Kay SL, Dock M (1995) Subgingival distribution of *Treponema denticola*, *Treponema socranskii*, and pathogen-related oral spirochetes: prevalence and relationship to periodontal status of sampled sites. J Periodontol 66:829–837
27. Sakamoto M, Takeuchi Y, Umeda M, Ishikawa I, Benno Y, Nakase T (1999) Detection of *Treponema socranskii* associated with human periodontitis by PCR. Microbiol Immunol 43:485–490
28. Sakamoto M, Takeuchi Y, Umeda M, Ishikawa I, Benno Y (2001) Rapid detection and quantification of five periodontophatic bacteria by real-time PCR. Microbiol Immunol 45:39–44
29. Sánchez-Jiménez MM, Cardona-Castro N (2004) Validation of a PCR for diagnosis of typhoid fever and salmonellosis by amplification of the hilA gene in clinical samples from Colombian patients. J Med Microbiol 53:875–878
30. Sela MN (2001) Role of *Treponema denticola* in periodontal diseases. Crit Rev Oral Biol Med 12:399–413
31. Sela MN, Krausz A, Steinberg D, Klinger A, Kohavi D (1999) Adherence of periodontopathic bacteria to bioabsorbable and non-absorbable barrier membranes in-vitro. Clin Oral Implants Res 10: 445–452
32. Seshadri R, Myers GSA, Tettelin H, Eisen JA, Heidelberg JF, Dodson RJ, Davidsen TM, DeBoy RT, Fouts DE, Haft DH, Selengut J, Ren Q, Brinkac LM, Madupu R, Kolonay J, Durkin SA, Daugherty SC, Shetty J, Shvarstbeyn A, Gebregeorgis E, Geer K, Tsegaye G, Malek J, Ayodeji B, Shatsman S, McLeod MP, Šmajs D, Howell JK, Pal S, Amin A, Vashisth P, McNeill TZ, Xiang Q, Sodergren E, Baca E, Weinstock GM, Norris SJ, Fraser CM, Paulsen IT (2004) Comparison of the genome of the oral pathogen *Treponema denticola* with other spirochete genomes. Proc Natl Acad Sci U S A 101:5646–5651
33. Simonson LG, Goodman CH, Bial JJ, Morton HE (1988) Quantitative relationship of *Treponema denticola* to severity of periodontal disease. Infect Immun 56:726–728
34. Takeuchi Y, Umeda M, Sakamoto M, Benno Y, Huang Y, Ishikawa I (2001) *Treponema socranskii*, *Treponema denticola*, and *Porphyromonas gingivalis* are associated with severity of periodontal tissue destruction. J Periodontol 72:1354–1363
35. Takeuchi Y, Umeda M, Ishizuka M, Huang Y, Ishikawa I (2003) Prevalence of periodontopathic bacteria in aggressive periodontitis patients in a Japanese population. J Periodontol 74:1460–1469
36. Tamura K, Nakano K, Hayashibara T, Nomura R, Fujita K, Shintani S, Ooshima T (2006) Distribution of 10 periodontal

bacteria in saliva samples from Japanese children and their mothers. Arch Oral Biol 51:371–377
37. Thomas DD (1996) Aspects of adherence of oral spirochetes. Crit Rev Oral Biol Med 12:399–413
38. Uitto V-J, Pan Y-M, Leung WK, Larjava H, Ellen RP, Finlay BB, McBride BC (1995) Cytopathic effects of *Treponema denticola* chymotrypsin-like proteinase on migrating and stratified epithelial cells. Infect Immun 63:3401–3410
39. Umeda M, Contreras A, Chen C, Bakker I, Slots J (1998) The utility of whole saliva to detect the oral presence of periodontopathic bacteria. J Periodontol 69:828–833
40. Umeda M, Chen C, Bakker I, Contreras A, Morrison JL, Slots J (1998) Risk indicator for harboring periodontal pathogens. J Periodontol 69:1111–1118
41. Umemoto T, Li M, Namikawa I (1997) Adherence of human oral spirochetes by collagen-binding proteins. Microbiol Immunol 41: 917–923
42. van Winkelhoff AJ, van der Velden U, Winkel EG, Graaff J (1986) Black-pigmented Bacteroides and motile organisms on oral mucosal surfaces in individuals with and without periodontal breakdown. J Periodontal Res 21:434–439
43. Watanabe K, Frommel TO (1996) *Porphyromonas gingivalis*, Actinobacillus actinomycetemcomitans and *Treponema denticola* detection in oral plaque samples using polymerase chain reaction. J Clin Periodontol 23:212–219
44. Yao ES, Lamont RJ, Leu SP, Weinberg A (1996) Interbacterial binding among strains of pathogenic and commensal oral bacterial species. Oral Microbiol Immunol 11:35–41