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Persistent Colonization with *Tannerella forsythensis* and Loss of Attachment in Adolescents

ABSTRACT

Colonization with *Tannerella forsythensis* may characterize the conversion of periodontally healthy sites into diseased sites. This three-year study describes the prevalence of *T. forsythensis* and its relationship to clinical loss of attachment (LOA) in a group of adolescents considered at risk of developing early chronic periodontitis. Adolescents with (LOA+) and without (LOA-) loss of attachment were examined at baseline and 1.5 and 3 yrs subsequently. On each occasion, attachment loss was measured on selected teeth, and the presence of *T. forsythensis* in their subgingival plaque samples was determined by PCR. *T. forsythensis* prevalence in LOA+ subjects at baseline (64%) increased to 82% and 86% on subsequent examinations. In contrast, prevalence of *T. forsythensis* in LOA- subjects was always significantly lower (25%, 36%, and 32%, respectively). The odds of loss of attachment were 8.16 times greater in subjects infected with *T. forsythensis* at each examination. These results suggest that *T. forsythensis* is strongly associated with loss of attachment in this adolescent population.

KEY WORDS: *Tannerella forsythensis*, attachment loss, adolescents, longitudinal, subgingival plaque, PCR.

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INTRODUCTION

It has been suggested that *Tannerella forsythensis* (formerly *Bacteroides forsythus*) is one of three oral pathogens that are important risk factors for the development of destructive periodontal disease in adults (Consensus report, 1996). However, few studies have examined the potential role for this micro-organism in early-onset forms of periodontitis (Darby and Curtis, 2001). Cross-sectional studies have demonstrated a higher prevalence of *T. forsythensis* in rapidly progressive periodontitis (Kamma *et al.*, 1995; Listgarten *et al.*, 1995). Similarly, the odds for a subject to have periodontitis were demonstrated to be 14 times greater when 5% or more of sampled sites harbored *T. forsythensis* (Haffajee *et al.*, 1998).

The nature of these studies—*i.e.*, the presence or absence of *T. forsythensis* in already-diseased subjects—precludes any determination as to whether the organism is a risk factor for the future development of disease. Recent longitudinal studies, in which subjects with little or no initial periodontal disease were followed for periods up to 5 yrs, have demonstrated that the presence of *T. forsythensis* characterized the conversion of periodontally healthy sites into diseased sites, as indicated by loss of attachment and loss of alveolar crest height (Tanner *et al.*, 1998; Machtei *et al.*, 1999; Tran *et al.*, 2001).

In England, some adolescents, particularly those of Indo-Pakistani origin and those of low educational and socio-economic status, have been shown to have a high risk of loss of connective tissue attachment and alveolar bone (Lennon and Davies, 1974; Clerehugh and Lennon, 1986). In this three-year longitudinal study, we describe the prevalence of persistent colonization with *T. forsythensis* in such a group. Correlating the pattern of colonization observed with loss of attachment may help elucidate the role of *T. forsythensis* in the etiology of tissue destruction in these subjects.

MATERIALS & METHODS

Subjects

The subjects of this study were chosen retrospectively from the participants of a three-year clinical trial that examined the effect of a triclosan/copolymer dentifrice on the incidence of periodontal attachment loss in adolescents (Ellwood *et al.*, 1998). Subjects were examined on three occasions (baseline, 1.5 and 3 yrs subsequently) at school by one trained and calibrated examiner (RPE). Schools had been selected to ensure a high percentage of Asian pupils originating from India, Bangladesh, and Pakistan, since these students were identified as being at risk of developing early chronic periodontitis. Written informed consent had been previously obtained from the parents or guardians of these students, who were enrolled in the greater Manchester area and aged from 11 to 13 yrs (mean 12.7 $\pm$ 0.33 yrs). Of the 480 subjects who completed the study, 24% recorded loss of attachment over the three-year period (n = 115). Of

these, only 28 who had completed all three examinations had sufficient plaque sample remaining available for microbiological analysis. Twenty-eight subjects who did not experience LOA over the three-year period (matched for dentifrice) were chosen as controls.

Clinical Investigations

We measured attachment loss to the nearest millimeter by identifying the cemento-enamel junction and measuring the distance to the base of the pocket using a Hu-Friedy PCP10 probe (Clerehugh and Lennon, 1984). Assessments were made at the mesio- and disto-buccal surfaces of all first molars and the upper left and lower right central and lateral incisors. For each subject, the sum of the loss of attachment at all sites was calculated.

Microbiological investigations were performed on pooled plaque samples from the mesio-buccal sites of both upper first molars. These sites had been previously demonstrated to be at high risk of developing early chronic periodontitis (Clerehugh *et al.*, 1995). Plaque was collected with the use of two paper-points that were inserted subgingivally for 10 sec. The paper-points were stored in vials containing 1 mL of sterile phosphate-buffered saline (PBS) with 0.01% thimerosal and several glass beads and kept frozen until microbiological analysis (Ellwood *et al.*, 1997).

Sample Processing

Microbiological analyses of the stored plaque samples from all three examinations were conducted concurrently at The University of Queensland. After being thawed, the paper-points in PBS buffer were vortexed for 15 sec, and a 0.5-mL quantity of the suspension was transferred to a 2-mL Eppendorf tube. After centrifugation for 4 min at 7000 *g* and 4°C, the pellet was first re-suspended in 100 μL of sterile water and then boiled for 15 min for disruption of the bacteria. Samples were cooled on ice for 5 min and again centrifuged at 7000 *g* and 4°C for 1 min for the elimination of cell debris.

Detection of T. forsythensis

A single-step polymerase chain-reaction (PCR) was used for the detection of *T. forsythensis* in the subgingival plaque samples. The method was essentially as described by Meurman *et al.* (1997) with some minor modifications. Primers were selected from the 16S ribosomal RNA sequence of *T. forsythensis* strain 338 (Genbank accession number L16495). The upper primer (5′ AAA ACA GGG GTT CCG CAT GG 3′) was identical to upper-strand bases 180-199, while the lower primer (5′ TTC ACC GCG GAC TTA ACA GC 3′) was identical to lower-strand bases 586-605.

A 5-μL aliquot of the sample supernatant was added to a 0.2-mL thin-walled PCR reaction tube (Quality Scientific Plastics, Petaluma, CA, USA) containing the PCR reaction mixture. The PCR reaction mix included 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Fisher Biotech, Perth, West Australia), 2.5 U DNA polymerase, 10 mM Tris-HCl [pH 8.3], 50 mM KCl, 2.5 mM $MgCl_2$ (Amplitaq Gold, Applied Biosystems, Foster City, CA, USA), and 0.3 μM of both primers (Genset Oligos, Genset Pacific Pty Ltd, Lismore, Australia) in a final volume of 50 μL. Amplification was carried out in a programmable thermal controller (PTC-100, MJ Research Inc., Watertown, MA, USA) with an initial cycle of 95°C for 2 min followed by 35 cycles of 95°C for 30 sec, 60°C for 1 min, and 72°C for 1 min. Final extension was at 72°C for 2 min. PCR aliquots (10 μL) were analyzed by agarose gel electrophoresis. Electrophoresis was carried out in 0.04 M Tris-acetate [pH 8.0], 0.001 M EDTA buffer at 60 V for 60 min in a 2% agarose gel containing ethidium bromide (0.5 μg/mL). The expected single 426-base-pair PCR product was visualized by UV light.

PCR Sensitivity and Specificity

The sensitivity of the PCR protocol was quantitated by analysis of DNA extracted from aliquots of a 10-fold serial dilution of a known concentration of *T. forsythensis* ATCC 43037. The limit of detection in this system was 10^3 bacteria (Fig. 1A).

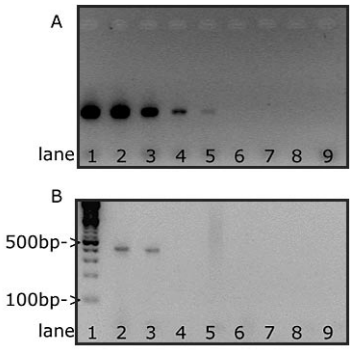

Figure 1. Sensitivity and specificity of the PCR methodology. **(A)** Lanes 1-8 show a serial dilution of *T. forsythensis* ATCC 43037 starting at 10^7 bacteria. Lane 9 is an assay-negative control (H_2O). The limit of sensitivity was estimated as 10^3 bacteria. **(B)** An agarose gel of PCR products of DNA extracted from two *T. forsythensis* strains and some of the panel of oral bacteria tested (not all negative results shown). Lane 1, DNA 100-base-pair marker; Lane 2, *T. forsythensis* ATCC 43037; Lane 3, *T. forsythensis* 7007; Lane 4, *A. actinomycetemcomitans* Y4; Lane 5, *A. actinomycetemcomitans* ATCC 29524; Lane 6, *P. intermedia* ATCC 25611; Lane 7, *P. intermedia* FDC 581; Lane 8, *P. gingivalis* FDC 381; and Lane 9, *P. gingivalis* ATCC 33277.

We examined the specificity of the primers used by attempting to amplify DNA extracted from a panel of oral bacteria. Species tested included: *T. forsythensis* ATCC 43037, *T. forsythensis* 7007, *Actinobacillus actinomycetemcomitans* Y4, *A. actinomycetemcomitans* ATCC 29524, *Prevotella intermedia* ATCC 25611, *P. intermedia* FDC 581, *Porphyromonas gingivalis* FDC 381, *P. gingivalis* ATCC 33277, *Fusobacterium nucleatum* ATCC 49258, *F. nucleatum* FDC 263, *Streptococcus sanguis* UQD 017, *Capnocytophaga gingivalis* ATCC 33124, *C. sputigena* ATCC 33123, *Haemophilus influenzae* UQD 130, *Actinomyces viscosus*, *Prevotella nigrescens* Q1164, *P. nigrescens* A5, and *Bacteroides fragilis* ATCC 25285. Only DNA from the *T. forsythensis* species was amplified, indicating a high degree of primer specificity (Fig. 1B).

Statistical Analysis

Subjects were grouped according to whether loss of attachment was observed (LOA+, 28 subjects) or absent (LOA-, 28 subjects). We determined LOA by examining the difference between the LOA measurements (maximum of 16 sites *per* subject) at baseline and after 3 yrs (Ellwood *et al.*, 1998). Pre-existing loss of attachment was noted in six subjects at baseline. Two of these gained attachment over the 3 yrs and were thus classified as LOA-. The remaining four subjects were classified as LOA+—three experiencing further LOA while one subject's level of LOA remained the same.

Binary logistic regression was used to examine the relationship between the presence or absence of LOA and *T. forsythensis* status. The significance of the differences between the proportions of subjects with detectable levels of *T. forsythensis* in LOA+ and LOA- groups was assessed by means of an approximate normal test for differences between proportions of two independent groups. The level of significance was set at $\alpha = 0.05$.

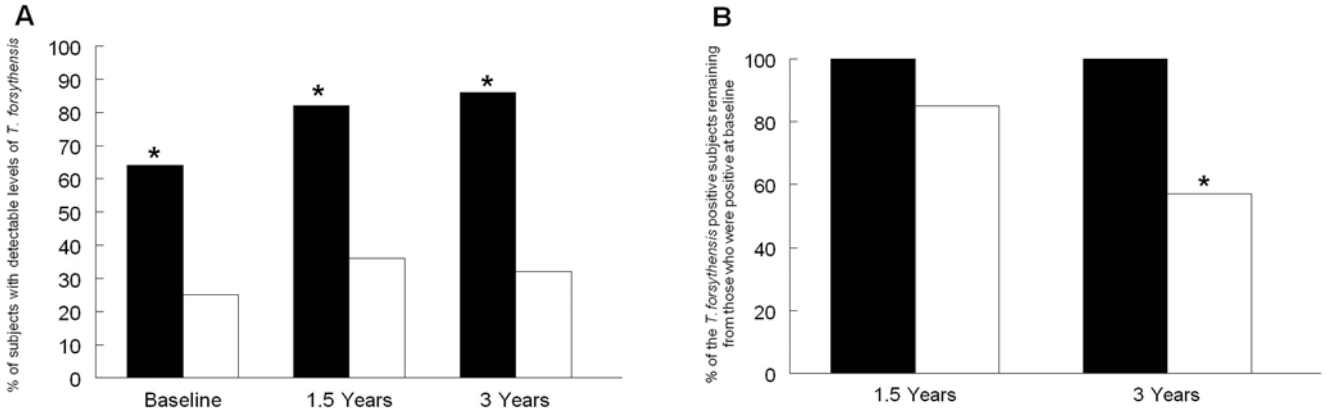

Figure 2. Prevalence and carriage rate of *T. forsythensis*. **(A)** The prevalence of *T. forsythensis* was significantly (*$p < 0.005$) higher in the LOA+ subjects (filled bars) compared with LOA- subjects (unfilled bars) at every examination (based on an approximate normal test for differences between proportions of two independent groups). The carriage rate of *T. forsythensis* in subjects who had detectable levels of *T. forsythensis* at baseline is demonstrated in panel **(B)**. Significantly (*$p < 0.005$) fewer (four of seven) LOA- subjects (unfilled bars) were demonstrated to maintain detectable levels of *T. forsythensis* longitudinally compared with 18 of 18 LOA+ subjects (filled bars).

RESULTS

Thirty-seven of the 56 subjects in this study harbored *T. forsythensis* at some stage during the 3 yrs. The organism was not detected at any examination in only 14% of LOA+ subjects compared with 53% of LOA- subjects ($p < 0.01$). In the LOA+ subjects, *T. forsythensis* was detected in 64% of the subjects at baseline. This prevalence increased to 82% and 86% on the second and third examinations. In contrast, the prevalence of *T. forsythensis* in the LOA- subjects was significantly lower on all 3 examinations (25%, 36%, and 32%, respectively, $p < 0.005$; Fig. 2A).

The carriage rates of *T. forsythensis* over the 3 yrs in both the LOA+ and LOA- subjects are shown in Fig. 2B. *T. forsythensis* colonization was maintained over the entire three-year period in all 18 LOA+ subjects who had *T. forsythensis* present at baseline. In the LOA+ subjects who acquired *T. forsythensis* post-baseline, the organism was then subsequently always present. In contrast, of seven LOA- subjects who also had *T. forsythensis* present at baseline, only four of these subjects maintained detectable levels of the organism ($p < 0.005$). Colonization in the LOA- subjects was also more volatile in comparison with the LOA+ subjects, *i.e.*, *T. forsythensis* was both gained and lost over the course of the 3 yrs.

There was a significant relationship ($p < 0.01$) between LOA over 3 yrs and the presence or absence of *T. forsythensis* at baseline. Over 3 yrs, the odds of LOA were much greater [odds ratio = 4.62; CI = (1.44, 13.79)] for the subjects with *T. forsythensis*. However, there was a stronger relationship ($p < 0.005$) between LOA and the number of visits that were positive for *T. forsythensis* over the 3 yrs. The odds of LOA in the 22 subjects with *T. forsythensis* present on all 3 examinations were 8.16 times greater [CI = (2.36, 28.21)] than for the 34 other subjects.

Within the LOA+ subjects, the mean attachment loss was shown to increase according to the period of *T. forsythensis* presence, but this did not reach significance.

Interestingly, of the 28 subjects with LOA+, 27 were of Indo-Pakistani ethnicity. By comparison, in the LOA- group, there were almost equal numbers of English (15) and Indo-Pakistani (13) subjects.

DISCUSSION

There is increasing evidence to implicate the Gram-negative anaerobe *T. forsythensis* in the pathogenesis of chronic periodontitis, and this has led to its inclusion in the list of recognized periodontal pathogens alongside *P. gingivalis* and *A. actinomycetemcomitans* (Consensus report, 1996).

Colonization of the oral cavity by many putative periodontopathogens can occur quite early in childhood without clinical signs of periodontal disease (Delaney and Kornman, 1987; Frisken *et al.*, 1987; Sweeney *et al.*, 1987; Conrads *et al.*, 1996). Recently, investigators have determined the prevalence of 10 oral organisms, including *T. forsythensis*, in children who showed negligible periodontal inflammation, in an attempt to delineate the prevalence and distribution of these pathogens in a 'healthy' population (Kimura *et al.*, 2002). Plaque from 144 healthy children aged 2 to 13 yrs (12 subjects from each year of age) was examined by means of a 16S rRNA-based PCR assay similar to that used in the present study. *T. forsythensis* was detected infrequently, especially in the younger children, but at least 10 to 20% of the children in each age group were infected with the organism.

In the present study, 45% of the adolescent population who initially had little or no periodontitis had detectable levels of *T. forsythensis* in their subgingival plaque. Furthermore, a strong association was observed between subjects with clinical loss of attachment over a three-year period and subjects with persistent, *i.e.*, sustained colonization by *T. forsythensis*. Previous longitudinal studies, albeit in adults with minimal periodontal disease, have been reported in the literature (Tanner *et al.*, 1998; Machtei *et al.*, 1999; Tran *et al.*, 2001). Examination of 415 subjects over a two-year period demonstrated that subjects who harbored *T. forsythensis* at baseline had greater loss of alveolar crest height and experienced greater proportions of sites losing attachment when compared with those who did not harbor *T. forsythensis* (Machtei *et al.*, 1999). Similarly, in our study, the presence of

T. forsythensis at baseline significantly increased the odds of loss of attachment over the 3 yrs (odds ratio = 4.62). In a recent study of 205 subjects, those positive for *T. forsythensis* at baseline had 3.7 times higher odds of having a site in their mouth losing attachment over a two-year period than subjects negative for *T. forsythensis* (Tran *et al.*, 2001). Significantly, these workers also reported that subjects with attachment loss tended to maintain the presence of *T. forsythensis* more consistently between visits. Indeed, when a subject had *T. forsythensis* present at all 5 examinations, the odds of his/her having a site that lost attachment increased to 5.3 when compared with those of a subject with occasional or no presence of *T. forsythensis*. These results are supported in our study, where subjects with *T. forsythensis* present at all 3 visits had 8.2 higher odds of having a site lose attachment over the 3 yrs.

In the present study, we made no attempt to try to relate the attachment loss observed at a particular site with its concurrent microbiology, since plaque from both examination sites was pooled for DNA analysis. This study, therefore, identified only which subjects were at greater risk, not which sites. Tanner *et al*. (1998), in a 12-month study of 56 healthy adults, demonstrated *T. forsythensis*, *Campylobacter rectus,* and *Selenomonas noxia* as the predominant species associated with active interproximal lesions. However, Tran *et al.* (2001) could not significantly associate any site-level descriptors with the presence of any pathogen, since *T. forsythensis*, *P. gingivalis*, and *A. actinomycetemcomitans* could be found frequently in plaque collected from interproximal areas of subjects with either mild or no periodontal disease.

The higher prevalence of persistent *T. forsythensis* observed in subjects of Indo-Pakistani ethnicity is similar to that reported earlier for *P. gingivalis* (Ellwood *et al.*, 1997). This may not be surprising, given the targeted cohort of the original study. Interestingly, since these subjects are genetically "Caucasian", this may suggest that other environmental factors may also influence detection rates in these subjects.

In summary, these results demonstrate that the persistent presence of *T. forsythensis* in subgingival plaque is strongly associated with loss of attachment in this at-risk adolescent population.

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