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The distribution of *Tannerella forsythia* in an adolescent and adult population

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Background: The fact that *Tannerella forsythia*, an important periopathogen, is difficult to cultivate from mixed infections has impeded precise estimates of its distribution within a given population. In order to discern *T. forsythia* alone from the mixed infection of plaque, the use of sensitive 16S ribosomal RNA based polymerase chain reaction (PCR) detection is necessary.

Objectives: The aim of the present study was to determine the distribution of *T. forsythia* in an adult and in an adolescent population.

Material and methods: Subgingival plaque samples were obtained from 498 Australian adults and from 228 adolescent subjects from Manchester, UK. *Tannerella forsythia* was detected using PCR and confirmed by restriction analysis. Semi-quantitation of the organisms was carried out using two specific primers of differing sensitivities.

Results: In the adolescent population, 25% were found to carry *T. forsythia*, albeit in relatively low numbers. In the adult population, a total of 37.8% and 11% were found to carry the organism with primer 2 and primer 1, respectively, suggesting that around 27% had between 10^3 and 10^7 organisms. Although there was an apparent increased proportion of *T. forsythia* positive subjects in those aged ≥ 50 years, this was not statistical significant. However, *T. forsythia* positive male smokers showed increased disease severity compared with *T. forsythia* negative subjects.

Conclusion: This study has shown that at least 25% of the adolescent population carry low numbers of *T. forsythia*, whereas at least 37% of adults carry the organism, with some 11% having relatively high numbers. The relationship between *T. forsythia* and disease progression in these populations, however, remains to be determined.

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It is generally accepted that around 10–15% of the world's population is affected with an advanced form of chronic periodontitis thought to be caused by plaques having aggressive clonotypes of *Porphyromonas gingivalis*, *Tannerella forsythia and Actinobacillus actinomycetomcomitans*. *Tannerella forsythia* (formerly called *Bacteroides forsythus*) has been associated with chronic periodontitis and is inherently a fastidious organism, which has impeded studies on its characterization (1). However, little is known of its distribution within either the wider adolescent or adult population (2). A small number of culture based identification studies of *T. forsythia* in adults have estimated that

around 53% have *T. forsythia* in disease, whereas only 24% of those in periodontal health have *T. forsythia* in their plaque (3). In a recent study using polymerase chain reaction (PCR), Umeda *et al.* (4), detected *T. forsythia* in 42.9% of children, with higher frequencies being found in females with a mixed dentition (85%). A cross-sectional study of adolescents of different ethnic origins in Greater Manchester, UK reported a statistically significant ($p > 0.001$) association between *P. gingivalis* and sites with attachment loss of > 3 mm (5). But the distribution of *T. forsythia* in the same adolescent population remains to be determined.

Recent cross-sectional and longitudinal studies have reported the distribution as well as acquisition and/or loss of putative periopathogens *A. actinomycetomcomitans*, *P. gingivalis* and *Prevotella intermedia* in a normal adult population (6, 7). However, data on *T. forsythia* and its natural distribution in normal adult populations remains scarce.

Culture and non-culture based bacterial identification studies in the past have encountered specificity limitations due to cross-reactivity or cross-hybridization with closely related species in a mixed periodontal infection. Recent studies have overcome this through the use of DNA-based identification methods that can discern closely related species (8, 9). Although PCR is the most sensitive detection method, it does have limitations when used on crude samples such as subgingival plaque. Heterogeneous DNA, hemoglobin, polysaccharides, and other biological molecules found in dental plaque may play a significant role in PCR amplification either by hampering DNA polymerases or by binding non-specifically to the oligoprimers, thus diminishing primer sensitivity (10). Hence, the impact of primer sensitivities in amplifying target DNA without cross-hybridization is vital when screening crude plaque samples. The aim of this study therefore is to use two different *T. forsythia* primers to detect and further elucidate the distribution of *T. forsythia* in two populations: (i) an adolescent population from greater Manchester, UK and (ii) an adult population from Brisbane, Australia.

Material and methods

Study subjects

Subgingival plaque samples of the same population from the Greater Manchester area that participated in the cross-sectional study by Ellwood *et al.* (5) was used for PCR assay. A total of 228 adolescents aged 11–13 years, with mean age 12.7 ± 0.33 years, were included in this study. Plaque was collected from the mesiobuccal surface of both maxillary permanent first molars (11).

The adult population was representative of a normal Australian adult population and was not a disease or 'at risk' group. Subgingival plaque samples of 498 employees of The University of Queensland, previously described (6, 7), aged between 18 and 65 years were used. Their mean age was 40 ± 10.8 years. This population comprised of an almost equal number of males and females. Plaque was pooled from 12 sites of each individual for PCR assay.

Plaque sampling

Briefly, in the adolescent population, sites were dried and plaque was collected with sterile paper points inserted to the most apical area of the gingival sulcus. In the adult population, following removal of supragingival plaque, subgingival plaque was collected using sterile curettes. Samples from both populations were stored in vials containing 1 ml of phosphate-buffered saline, 0.01% thiomersol and glass beads, and stored at –80°C till PCR analysis.

None of the participants in either population had a history of any relevant systemic disease, nor were they on antibiotic therapy. Appropriate ethical clearance with written consent was obtained from Human Ethics Review Committees of the University of Manchester and the University of Queensland.

Cultivation of *Tannerella forsythia*

Tannerella forsythia ATCC 43037^T was grown in brain heart infusion/peptone media in a 500-ml culture containing agar 5.5 g, brain heart infusion 18.5 g, tryptone/peptone 5 g, yeast 1.5 g, menadione 0.5 mg/ml (Vitamin K), haemin 1 mg/ml and 5% defibrinated sheep blood. The bacterium was innoculated on to a plate with a disk containing 300 mg of *N*-acetylmuramic acid and cocultured with *Streptococcus aureus* as a feeder. The plates were incubated for 6–7 days at 37°C in an anaerobic jar containing 80% nitrogen, 10% carbon dioxide and 10% hydrogen.

Cultivation of *Porphyromonas gingivalis*

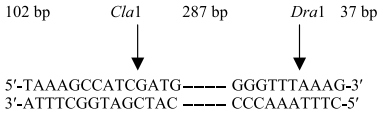
Porphyromonas gingivalis ATCC 33277^T was grown in brain heart infusion broth supplemented with yeast extract (0.3%), trypticase peptone (1.0%), sodium bicarbonate (0.2%), hemin (1 mg/ml) and manadione (0.5 mg/ml) at 37°C in an atmosphere of 80% nitrogen, 10% carbon dioxide and 10% hydrogen in an anaerobic cabinet (12).

Primer specificity

Specificity of *T. forsythia* specific primers was determined by amplification of a panel of subgingival species, reference strains including *T. forsythia*, *P. gingivalis*, *Fusobacterium nucleatum*, *A. actinomycetomcomitans* and *P. intermedia*, with *T. forsythia* specific primers: primer 1 (F) 5′-GCGTATGTAACCTGCCCGCA-3′; (R) 5′-TGCTTCAGTGTCAGTTATACCT-3′, amplicon 641 bp (8); Primer 2 (F) 5′-AAAACAGGGGTTCCGCATGG-3′; (R) 5′-TTCACCGCGGACTTAACAGC-3′, amplicon 426 bp (9); and ubiquitous primer (F) 5′-GATTAGATACCCTGGTAGTCCAC-3′; (R) 5′-CCCGGGAACGTATTCACCG-3′, amplicon 602 bp (8). Further, specificity of the PCR amplicons of the adult population was ascertained by restriction analysis (see below).

Restriction mix

The PCR amplicons were restricted with *Cla*1 and *Dra*1 enzymes (Promega Corporation, Madison WI, USA) with expected amplicons of 102 bp, 287 bp and 37 bp. The restriction mix consisted of 5 μl purified DNA, 2 μl *Cla*1, 2 μl *Dra*1, 4 μl buffer B, 0.2 μl bovine serum albumin and 6.8 μl water. This mix was incubated at 37°C for 1 h and further electrophoresed at 50 V on 12% polyacrylamide gel.



Polymerase chain reaction protocol

The samples were brought to 37°C, vortexed well and 0.2 ml of each sample was pelleted and washed three times with distilled water. The pellet was resuspended in 0.1 ml distilled water, boiled for 10 min and placed on ice. This was centrifuged at 10,000 ***g*** in a microfuge for 10 min. The supernatant containing total DNA of plaque sample was used for PCR analysis (8).

All the samples were screened separately by each specific primer and an ubiquitous primer common to all Gram-negative bacteria. The PCR reaction was set up by adding 5 μl of the supernatant that contained the DNA to 45 μl of reaction mixture containing 5 μl of 10 × PCR buffer; 1.25 U of T*th* polymerase; 0.2 mM of each deoxyribonucleotide; 1.5 mM of $MgCl_2$ (Fisher Biotech, Fisher Scientific Inc., Springfield, NJ, USA), and 0.3 μM each of specific primers 1 and 2 and ubiquitous primer for every individual sample. PCR amplification was performed on a thermocycler (PTC-100_{TM}, M.J. Research, Inc., Waltham, MA, USA), using the following cycle conditions. Initial denaturation at 95°C for 2 min, followed by 36 cycles of denaturation at 95°C for 30 s, primer annealing at 60°C for 1 min, followed by extension at 72°C for 1 min. A final end fill at 72°C for 2 min was followed by a 4°C hold. PCR products were electrophoresed on 2% agarose gel, containing 0.5 μg/ml ethidium bromide at 4 V/cm, viewed under UV transillumination.

All assays included a positive control (*T. forsythia* ATCC 43037^T), a negative control (blank with no template DNA) and a closely related species (*P. gingivalis* 33277^T) for cross-hybridisation check with other bacteria in plaque.

Spiking assay: establishing sensitivity of the *Tannerella forsythia* specific primers

A 10 × serial dilution of *T. forsythia* pure culture with an initial concentration of 1×10^9 cells/ml was subjected to DNA extraction, similar to that followed for plaque samples. Then 5 μl DNA of each serial dilution of pure culture and 5 μl DNA of each *T. forsythia* negative clinical samples were added to individual 40 μl reaction mix containing individual *T. forsythia* specific primer 1 and primer 2 and amplified.

Detection of *Tannerella forsythia* in subgingival plaque of adolescents and adults

A total of 228 (two sites per individual) adolescent samples and 498 adult samples (12 sites per individual) were subjected for PCR assay. The samples were pooled for each individual. Of this, 200 μl was used for DNA extraction. Both the populations were screened by PCR with the two *T. forsythia* specific primers: primer 1 (8) and primer 2 (9).

Statistical analysis

Using the SAS system FREQ procedure the chi-squared analysis was done to obtain significant association with presence of *T. forsythia* and periodontal status of all the subjects. A forward stepwise logistic regression procedure, SAS LOGISTIC, was used to assess relative risk for those variables with a significance level limit of 0.05. Further, associations with severity of the disease using the stepwise analysis were applied. The odds ratio and 95% confidence interval of association with smoking and gender to severity of the disease was obtained.

Extent of the disease

To determine the extent of the disease in the population, two, four, six and eight sites with a threshold limit of 4 mm probing depth were used.

Severity of the disease

To estimate the severity of the disease, subjects were identified with two or more sites with probing depth ≥ 3 mm to < 4 mm, ≥ 4 mm to < 5 mm, ≥ 5 mm to < 6 mm and ≥ 6 mm.

Association of age and *Tannerella forsythia* positive subjects

The 498 adult subjects PCR screened with primer 1 and primer 2 were divided into three age groups: < 35 years, 35–50 years, and > 50 years. Percentages of *T. forsythia* positive subjects within each of these age groups were calculated. The Pearson chi-squared test was performed using SPSS statistical package to test for an association between age and level of *T. forsythia*.

Association of smoking and *Tannerella forsythia* positive subjects

Smokers and non-smokers of both genders who were *T. forsythia* positive were analysed to test for an association between the extent and severity of the disease.

Results

Specificity and cross-hybridization

The specificity of the *T. forsythia* specific primers tested against a panel of subgingival species and the plaque samples of both the populations showed no non-specific PCR products or cross-hybridization. In addition, restriction analysis of the PCR amplicons (426 bp) from the adult plaque samples yielded 102 bp, 287 bp and 37 bp, thus confirming the *T. forsythia* specificity (Figs 1A and B). The 37-bp size was too small to retain on the gel (not seen).

Sensitivities of *Tannerella forsythia* primers: spiking assays

Sensitivity testing showed that with pure culture primer 1 detected 10^5 cells/ml (Fig. 2A1), whereas the results of the

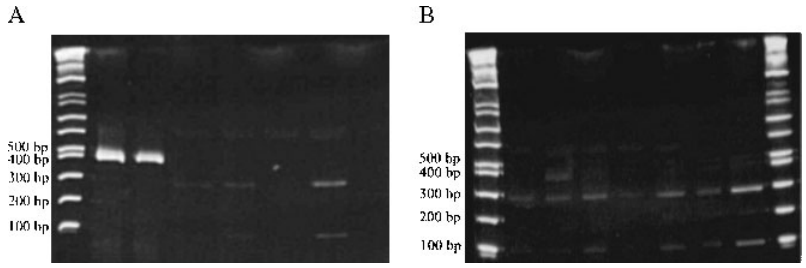


Fig. 1. Restriction analysis of polymerase chain reaction (PCR) product (amplicons 426 bp): (A) Lane 2, unrestricted amplicon of *Tannerella forsythia* pure culture; lane 3, unrestricted PCR amplicon of a clinical sample. The other lanes of (A) and (B) are restricted amplicons of clinical samples.

spiking assay showed that this primer could detect 10^7cells/ml (Fig. 2A2). On the other hand, primer 2 showed a very high sensitivity of 10^3 cells/ml, even when spiked with *T. forsythia* negative clinical samples (Figs 2B1 and B2). There was no inhibition of amplification by extraneous DNA or any biological material that could be present in the supernatant. The ubiquitous primer amplified all the serial dilutions by both the specific primers. This differential sensitivity allowed the distribution of *T. forsythia* to be determined as negative or $< 10^3$, $> 10^3$ but $< 10^7$, and $> 10^7$.

Detection of *Tannerella forsythia* in subgingival plaque of adolescents and adults

Subgingival plaque of the adolescent population showed *T. forsythia* positive in 57/228 (25%) using primer 2 and 0% using primer 1. The adult population showed *T. forsythia* positive in 55/498 (11%) with primer 1 and 188/498 (37.8%) with primer 2 (Figs 3A and B). The ubiquitous primer amplified all the samples in both the populations.

Distribution of *Tannerella forsythia* among various age groups

The distribution of *T. forsythia* within each age of the adult population is shown in Table 1. It can be seen that the highest proportion of subjects harbouring *T. forsythia* are in the group aged 50 years and above. The greatest proportion of subjects with the high levels of *T. forsythia* is also within this group, although the differences between the groups did not reach statistical significance.

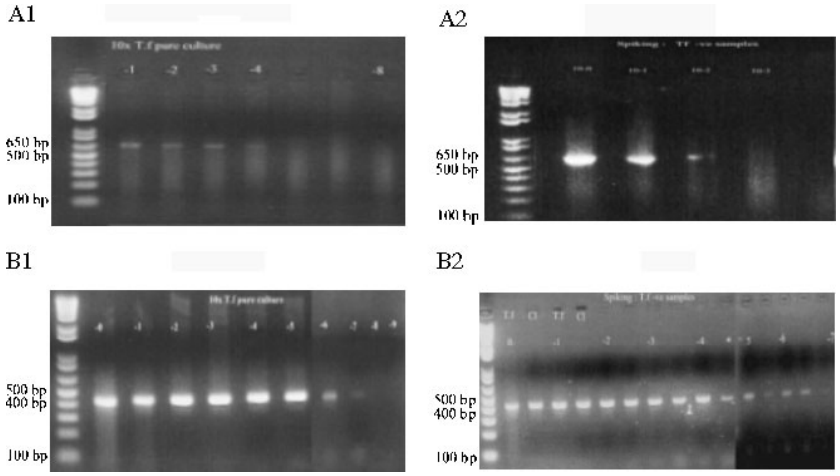


Fig. 2. Spiking assay: *Tannerella forsythia* negative clinical sample spiked by pure culture $10 \times$ dilution of *T. forsythia* ATCC 43037^T. (A1) Lane 1, molecular size marker (1-kb DNA ladder); lanes 2–10, $10 \times$ serial dilution of *T. forsythia* pure culture by specific primer 1. (A2) Negative clinical samples spiked with pure culture dilutions. (B1) Lanes 2–11, $10 \times$ serial dilution of *T. forsythia* pure culture by specific primer 2. (B2) Negative clinical samples spiked with pure culture dilutions.

Extent of the disease in *Tannerella forsythia* positive population

The presence of *T. forsythia* in relation to the set periodontal attachment threshold limit of probing depth of 4 mm showed that an increasing percentage of subjects had *T. forsythia* with an increasing number of sites, with a maximum of six sites but with less than eight sites being the most significant (56%) compared to all the other groups (Fig. 4).

Severity of the disease in *Tannerella forsythia* positive population

Having considered the threshold limit as two or more sites, the percentage of all *T. forsythia* positive subjects increased with deeper probing depths, with the maximum of 58.7% in probing depth ≥ 5 mm to < 6 mm (Fig. 5).

Relationship of gender and extent of the disease

The percentage of males having *T. forsythia* gradually increased from four sites ≥ 4 mm (39%) by ~8–9% with every two-site increase. However, the reverse was true in females, where the percentage gradually decreased by ~10% from a maximum at four sites ≥ 4 mm (58%). Females had significantly more *T. forsythia* (1.4 times) than males at ≥ 4 sites, whereas males had more than females (1.4 times) at ≥ 8 sites ($p < 0.05$) (Fig. 6).

Relationship of gender and severity of the disease

There was an almost equal distribution in the percentage of males harbouring *T. forsythia* with increased probing depths. A maximum of 66.7% had *T. forsythia* with two or more sites ≥ 6 mm to < 7 mm. On the contrary, there was a decrease of more than 10% in females with two or more sites ≥ 6 mm to < 7 mm compared with other groups (Fig. 7).

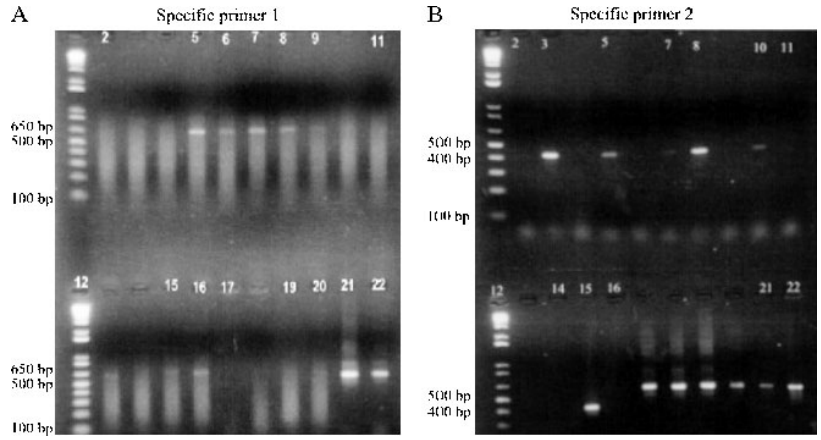


Fig. 3. Polymerase chain reaction (PCR) of subgingival samples of adult population using *Tannerella forsythia* specific primers. (A) Specific primer 1 (amplicon 641 bp). Upper panel: clinical samples with specific primer, showing both *T. forsythia* positive and *T. forsythia* negative samples. Lower panel: lane 15, positive control, *T. forsythia*; lanes 14 and 16, negative controls, *Porphyromonas gingivalis* (cross-hybridization check) and blank (no template), respectively; lanes 17–20 are the same clinical samples with ubiquitous primer. (B) Specific primer 2 (amplicon 426 bp). Upper panel: clinical samples with specific primer, showing both *T. forsythia* positive and *T. forsythia* negative samples. Lower panel: lane 15, positive control, *T. forsythia*; lanes 14 and 16, negative controls, *P. gingivalis* (cross-hybridization check) and blank (no template), respectively; lanes 17–20 are the same clinical samples with ubiquitous primer.

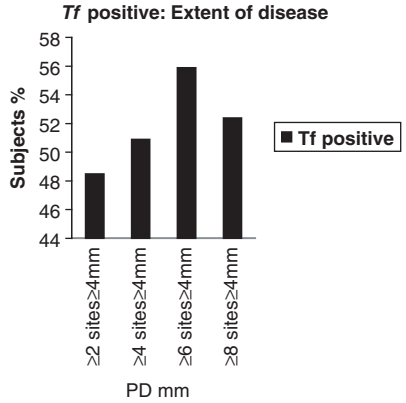


Fig. 4. Extent of the disease. Proportion of subjects positive with *Tannerella forsythia* in each disease category with a threshold limit of probing depth = 4 mm ($n = 498$) ($p < 0.05$).

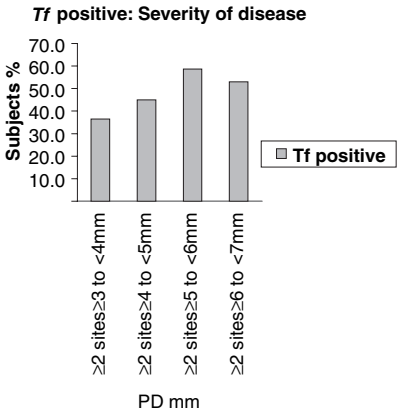


Fig. 5. Severity of the disease. Proportion of subjects in each disease category positive with *Tannerella forsythia* ($n = 498$).

Relationship of smoking and extent of the disease

The percentage of male smokers having *T. forsythia* steadily increased with increasing number of sites by approximately 1.5–1.7 times, with a maximum at ≥ 8 sites ≥ 4 mm ($p < 0.05$). On the contrary, the percentage of female smokers having *T. forsythia* was consistently low irrespective of number of sites, except at ≥ 4 sites (Fig. 8).

Relationship of smoking and severity of the disease

The male smokers harbouring *T. forsythia* had increased severity of disease, with the percentage gradually increasing with deeper probing depths. The female smokers did not show any association with severity of disease (Fig. 9).

Table 1. Distribution of *Tannerella forsythia* among various age groups

	T. forsythia			
Adults ($n = 498$) (age group)	$< 10^3$cells/ml 63%	10^3–10^7cells/ml 29.4%	$> 10^7$ cells/ml 7.6%	Total % 100%
T. forsythia levels cross-tabulation				
< 35 count	117	50	11	178
% within age group	65.6%	28.0%	6.4%	100%
35 age < 50 count	150	69	16	235
% within age group	63.7%	29.5%	6.8%	100%
≥ 50 count	48	27	10	85
% within age group	56.0%	32.0%	12.0%	100%

There is no significant association between *T. forsythia* levels and increasing age ($p > 0.05$).

Discussion

Previous data using a culture-based detection method have shown that around 24% of periodontally healthy subjects are *T. forsythia* positive (3). In the present study, however, around 37.8% of the adult population were shown to be *T. forsythia* positive, of which the majority were > 50 years of age. This could partly be due to the high sensitivity of the PCR assay, together with the fact that around 10% of the subjects had significant periodontal disease.

A sensitivity discrepancy of the two specific primers in detecting *T. forsythia* was seen in both the adolescent

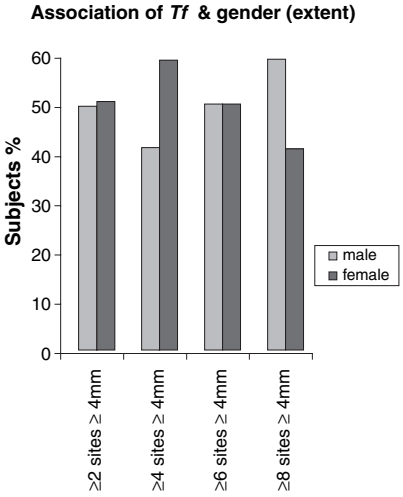


Fig. 6. Gender association to extent of disease in subjects having *Tannerella forsythia* in plaque. The percentage of males having *T. forsythia* increases by ~8–9% with every subsequent increase of two sites, whereas females having *T. forsythia* decreased by ~10% with increased number of sites and were statistically significant ($p < 0.05$).

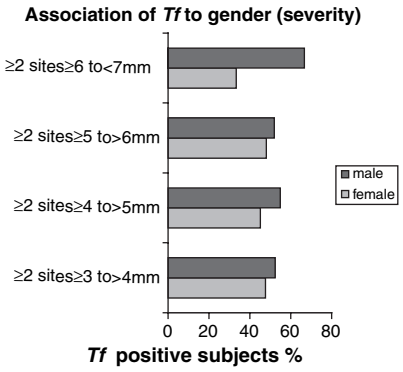


Fig. 7. Gender association with disease severity in subjects having *Tannerella forsythia* in plaque. Percentage of females having *T. forsythia* decreased with increased probing depth. The percentage of males having *T. forsythia* increased with increasing probing depths, with the percentage reaching a maximum of 66.7%.

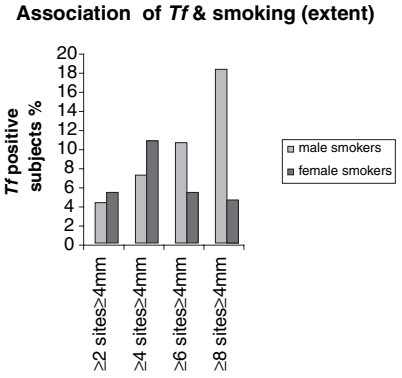


Fig. 8. Association of smoking to extent of the disease in subjects having *Tannerella forsythia* in plaque. There is a significant increase in percentage of males having *T. forsythia* with increasing number of sites ($p < 0.05$), whereas the female percentage is almost consistent except in ≥ 4 sites ≥ 4 mm, though not significant in attachment loss.

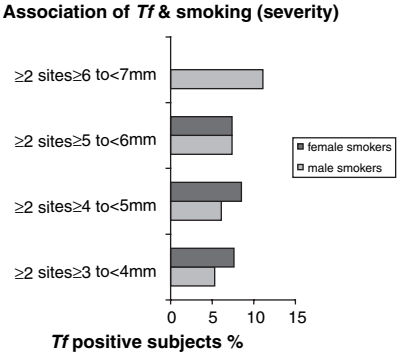


Fig. 9. Association of smoking and severity of disease in subjects having *Tannerella forsythia.* Smoking males had increased probing depths, whereas there was no particular association of female smokers habouring *T. forsythia* and disease severity.

and adult populations. The results using primer 2 showed that *T. forsythia* occurs in around 25% of the normal adolescent population, albeit in small numbers, and in 37.8% of the normal adult population. The results with primer 1 further showed that about 11% of the adults have relatively high numbers of the organism. This sensitivity difference between the two primers indicates that although the specific primers do amplify the target 16Sr RNA, they do so with the differential amplification rates, most likely dictated by the type of sample and its processing conditions.

The validity of the results of a PCR amplification of a 'target' organism, particularly of a mixed infection, is confirmed by ascertaining absence of cross-hybridization especially with phylogenetically closely related species. Although subgingival plaque contains numerous species, the two *T. forsythia* primers did not show cross-hybridization with the phylogenetically closely related species *P. gingivalis* or with the panel of oral organisms used. The absence of non-specific PCR products and the restriction analysis of the PCR amplicons confirmed that both the primers were indeed *T. forsythia* specific. However, under the conditions used, the sensitivity of the two primers differed considerably. This may have been due to the extraneous DNAs of other subgingival species in the supernatant in addition to the 16Sr DNA of *T. forsythia*. Also, several other biologic molecules that are likely to interfere in PCR amplification could have played a role in the diminished primer sensitivity (10). This could explain why in the present study primer 1 detected 10^5 cells in pure samples but in the spiked bacterial samples (spiking assay) the primer had a detection limit of only 10^7 cells/ml. On the other hand, primer 2, with greater sensitivity, amplified as few as 1×10^3 cells/ml, with little or no impediment by extraneous DNA, when compared to primer 1. This could be explained by a possible mispriming (at competitive primer annealing sites) the effect of other biological molecules, or a cumulative effect of either could impact on PCR amplification and cannot be ruled out.

The discrepancy in sensitivity between the two primers allowed an estimate of the number of *T. forsythia* in the individual samples. In this context, the 25% *T. forsythia* positive adolescents detected using primer 2 and none using primer 1 would seem to suggest that these adolescents carried *T. forsythia* in low levels of between 10^3 and 10^7. In a similar age group using primer 1, Ashimoto *et al.* reported a prevalence of *T. forsythia* of around 8% (8). Recently, Umeda *et al.* (4) showed that around 43% of children aged 1–15 carried *T. forsythia*. Of interest in this Japanese population was the fact that higher frequencies were found in females with mixed dentition and who had poor oral hygiene. The authors (4) speculate that this may be due the difficulty in maintaining oral hygiene in the mixed

dentition, as well as to hormonal changes seen in females.

In the adult population, the 'primer' sensitivities again showed a clear difference. The highly sensitive primer 2 identified 37.8% of the adult population to be positive for *T. forsythia*. At the same time, however, 11% of the population were shown to be positive using the less sensitive primer 1, suggesting that these subjects had relatively high numbers of *T. forsythia* ($> 10^7$ cells/ml) (8). Therefore, the results of the present study suggest that mere presence of bacteria may not reflect the periodontal status of this population.

Although approximately 50% of *T. forsythia* positive subjects in this population were in the 50–65 years age group, no association could be found between the different age groups in terms of the prevalence of this organism, indicating that increasing age has minimal or no influence. Smokers were 1.5 times more likely to have *T. forsythia* compared with non-smokers. Other studies (13) also have shown that smokers harbour significantly higher levels of *T. forsythia* than non-smokers and were 2.3 times more at risk than non-smokers. The distribution of male smokers with *T. forsythia* in particular showed a linear correlation to the number of sites affected. Similar studies (14) have shown smokers to have a higher proportion of deep pockets probing depth > 5 mm.

The results of the present study have shown that a significant proportion of the normal adult and adolescent populations carry *T. forsythia*. However, the relationship between the presence of *T. forsythia* (together with other periodontal pathogens) and future disease progression needs to be ascertained.

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