Prevalence of Periodontal Pathogens in Dental Plaque of Children

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Porphyromonas gingivalis, *Actinobacillus actinomycetemcomitans*, and *Tannerella forsythensis* have been implicated as the main etiological agents of periodontal disease. The purpose of this work was to estimate the prevalence of these organisms in plaque from children without gingivitis (group 1; $n = 65$) and from those with gingivitis (group 2; $n = 53$). Extracted DNA from plaque was subjected to two rounds of PCR targeting the 16S rRNA gene using both universal primers and species-specific primers. The results were as follows: group 1, *P. gingivalis*, 49%; *A. actinomycetemcomitans*, 55%; and *T. forsythensis*, 65%; group 2, *P. gingivalis*, 47%; *A. actinomycetemcomitans*, 59%; and *T. forsythensis*, 45%. *T. forsythensis* was detected more frequently in children with no gingivitis than in those with gingivitis ($P = 0.03$). There was no significant difference between the two groups with respect to the presence of *P. gingivalis* or *A. actinomycetemcomitans* in either group ($P > 0.05$). Logistic regression analysis revealed that the odds of a patient having gingivitis were 2.3 times greater in the absence of *T. forsythensis*. In conclusion, the results of this study have shown that the three pathogens can be detected in the dental plaque of healthy children and of those with gingivitis and that *T. forsythensis* is associated with dental plaque at sites with no gingivitis.

The continuous accumulation of dental plaque as a result of impaired or poor oral hygiene results in gingivitis. This inflammation occurs as a direct response to bacteria in the plaque (4). Gingivitis is a reversible condition, and while this inflammation may precede periodontitis, not all cases of gingivitis will progress to periodontitis (2). Clinical studies have revealed the presence of 10 to 15 bacterial species that are potential periodontal pathogens in adults (7, 26); of these, the three most cited are *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, and *Tannerella forsythensis* (formerly *Bacteroides forsythus*), which have been implicated in the progress of periodontal diseases (1, 16, 23, 30). There are few data concerning the prevalence of *P. gingivalis*, *A. actinomycetemcomitans*, and *T. forsythensis* in the plaque of healthy children. The available prevalence data for these pathogens are also contradictory; for example, *P. gingivalis* has been isolated from the plaque of 80% of children during and after puberty (28), whereas other studies failed to detect *P. gingivalis* in prepubertal children (9, 31). Although periodontal disease is rare in healthy children, it is important to investigate the presence of periodontal pathogens as the permanent teeth start to erupt. The detection of periodontal pathogens before puberty may be helpful in identifying which children need more effective oral health programs in order to minimize the risk of periodontal disease after puberty (27).

The introduction of molecular techniques, particularly nested PCR, has lowered the routine threshold of bacterial detection to as few as 10 cells (24). A PCR technique involving a set of primers that target species-specific regions of the 16S rRNA genes of *P. gingivalis*, *A. actinomycetemcomitans*, and *T. forsythensis* has been developed to demonstrate the presence of

these periodontal pathogens in adults (24). This paper presents an adaptation of this protocol to study the prevalence of the three periodontal pathogens in plaque samples taken from healthy prepubertal children aged between 5 and 9 years, with and without gingivitis.

MATERIALS AND METHODS

Ethical approval (Joint Research and Ethics Committee reference no. 00/E031) was obtained from the Eastman Dental Hospital National Health Service Trust, London, United Kingdom. Each parent was given an information sheet to read and asked for written consent. Each child was asked for verbal consent.

Subjects. Healthy children aged between 5 and 9 years being treated at the Department of Paediatric Dentistry and the School of Dental Therapy at the Eastman Dental Hospital were recruited. Children with chronic medical disorders or who had been treated with antibiotics within the preceding 3 months were excluded. Two groups of children were selected: group 1, children with discernible plaque but no gingivitis associated with either the lower left or lower right first permanent molar tooth, and group 2, children with discernible plaque and gingivitis associated with either the lower left or lower right first permanent molar tooth. Plaque and gingivitis scores were recorded for each child using a modification of the method of O'Leary (5). Four gingivally related quadrisections of each tooth (mesiobuccal, distobuccal, mesiolingual, and distolingual) were visually examined to give a score of 0 for no discernible plaque and 1 for discernible bacterial dental plaque deposits. Similarly, each tooth quadrisection associated with no gingival inflammation was given a score of 0 or a score of 1 for gingival inflammation.

Dental plaque was sampled from the buccal and lingual gingival crevices of either the lower left or lower right first permanent molar tooth using a sterile wooden stick. Where both lower permanent first molars were erupted, either the right or left tooth was randomly selected. The wooden stick was immediately placed in a sterile container with 1 ml of reduced transport fluid (19) and five glass beads. The plaque samples were dispersed in the reduced transport fluid by vortexing them for 10 s, and the whole genomic DNA was immediately extracted using the Puregene DNA isolation kit for yeast and gram-positive bacteria (Gentra Systems, Minneapolis, Minn.). The genomic DNA was then stored at -20°C .

Plaque sampling and sample size calculation. The detection of *A. actinomycetemcomitans* is more frequent in children with discernible gingivitis than in children with no discernible gingivitis (9). We expected to detect the organism in 50% of children with no discernible gingivitis. In children with discernible gingivitis, a difference in the detection frequency of *A. actinomycetemcomitans* of $>30\%$ would be microbiologically and possibly clinically significant. A two-group

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continuity-corrected chi-square test with a 0.05 two-sided significance level has 80% power to detect the difference between a discernible gingivitis group proportion, 0.800, and a nondiscernible group proportion, 0.500, when the sample size in each group is 45.

Genomic DNA preparation of reference strains from culture. The strains *P. gingivalis* NCTC 11834 and *A. actinomycetemcomitans* NCTC 9710 were obtained from the Public Health Laboratory Services (Colindale, United Kingdom). The strain *T. forsythensis* ATCC 43037 was obtained from the American Type Culture Collection (ATCC) (Manassas, Va.). *P. gingivalis* was grown on fastidious anaerobic agar (Lab M, Bury, United Kingdom) supplemented with 5% defibrinated horse blood (E&O Laboratories, Bonnybridge, United Kingdom), 10 µg of hemin (Sigma, Poole, United Kingdom)/ml, and 1 µg of vitamin K₁ (Sigma)/ml. *A. actinomycetemcomitans* was grown on tryptone soy agar (Oxoid, Basingstoke, United Kingdom) supplemented with 6 g of yeast extract (Oxoid)/liter. After being autoclaved, the medium was further supplemented with 0.15% glucose and 0.4% NaHCO₃ (18). *T. forsythensis* was grown on tryptone soy agar (Oxoid) supplemented with 5% defibrinated horse blood, 4 g of yeast extract/liter, and 10 mg of *N*-acetylmuramic acid (Sigma)/liter (20). The strains were incubated in an anaerobic chamber (Don Whitley Scientific Ltd., Shipley, West Yorkshire, United Kingdom) with an atmosphere containing 80% nitrogen, 10% hydrogen, and 10% carbon dioxide at 37°C for 7 days.

Approximately two or three colonies of each reference strain were transferred into nutrient broth. *P. gingivalis* was grown in Todd-Hewitt broth (Oxoid) supplemented with 10 µg of hemin/ml and 1 µg of vitamin K₁/ml. *A. actinomycetemcomitans* was grown in tryptone soy broth supplemented with 6 g of yeast extract/liter; after being autoclaved, the medium was further supplemented with 0.15% glucose and 0.4% NaHCO₃. *T. forsythensis* was grown in tryptone soy broth supplemented with 4 g of yeast extract/liter and 10 mg of *N*-acetylmuramic acid/liter. The broths were then incubated anaerobically for 3 days. A whole-genomic DNA extraction from 1 ml of broth culture was obtained using the Puregene DNA isolation kit.

PCR primers. The whole-genomic DNA extracts from plaque were used as templates in a PCR using the universal primers that target the 16S rRNA gene. The nucleotide sequences of the forward and reverse primers were 5'-AGAGT TTGATCMTGGCTCAG-3' (27F) and 5'-TACGGYTACCTTGTTACGACT T-3' (1492R) (11). The expected product length for this PCR was ca. 1,500 bp, and this was compared with a DNA molecular size marker (PCR Marker; Amresco, Solon, Ohio). The primers chosen for the detection of the three putative pathogens targeted specific regions of the 16S rRNA gene. These consisted of three forward primers and one conserved reverse primer. The expected product lengths were 197 bp for *P. gingivalis*, 360 bp for *A. actinomycetemcomitans*, and 745 bp for *T. forsythensis*. These were compared with a DNA molecular size marker (100-bp molecular size marker; Promega, Southampton, United Kingdom). The nucleotide sequences for the four selected primers were as follows: *P. gingivalis*-specific forward primer (PgF), 5'-TGATAGTACTGATG GTGAAAACC-3'; *A. actinomycetemcomitans*-specific forward primer (AaF), 5'-ATTGGGGTTTATGCCCTGGTG-3'; *T. forsythensis*-specific forward primer (BfF), 5'-TACAGGGGAATAAATGAGATACG-3'; and conserved reverse primer (C11R), 5'-ACGTCATCCCCACCTTCCTC-3'. (All primers were obtained from Genosys, Haverhill, Cambridgeshire, United Kingdom.)

Determination of detection limit. The lowest detection limit for each of the target strains was determined by both one round of PCR using universal primers and two rounds of PCR using the specific nested primers. This was achieved with 10-fold dilutions of pure cultures of the type strains. The dilutions were based on microscopic counts using a hemocytometer. Numbers ranging from 1 to 10⁸ cells/ml for each species were prepared, and a whole-genomic DNA extraction was carried out. In order to ensure the precipitation of the entire bacterial DNA, carrier DNA (15) was used. The carrier DNA was 10 µg of salmon sperm DNA (Sigma). The nested PCR was carried out for the three pathogens individually in the presence of 10 ng of genomic DNA/µl extracted from *Capnocytophaga ochracea* ATCC 27872. The positive controls in both rounds of PCR consisted of 10 ng of type strain DNA/µl. A further positive control consisting of 10 ng of *C. ochracea* DNA/µl was also included in the nested PCR. This reaction required the use of 0.2 µM universal forward primer (357F), 5'-CCTACGGGAGGCAG CAG-3', and 0.2 µM *Capnocytophaga* genus-specific reverse primer (562R), 5'-CCCTTTAAACCAATGAT-3'. Two negative controls consisting of 10 ng of *C. ochracea* DNA/µl and 0.01 µg of salmon sperm DNA/µl were included. The positive and negative controls from the first round of PCR were also used as templates in the nested multiplex PCR.

PCR with universal primers. The final volume of each PCR mixture was 100 µl. The amplification reaction mixture contained 5 mM 10× NH₄ buffer, 2.5 mM MgCl₂, 0.2 µM (each) primers 27F and 1492R, and 5 U of *Taq* polymerase (BioTaq; Bionline, London, United Kingdom). All deoxynucleoside triphosphates

(dNTPs) (Promega) were used at a final concentration of 0.2 mM. The cycling parameters carried out on a Primus thermal cycler (MWG Biotech, Milton Keynes, United Kingdom) consisted of 30 cycles of 94°C for 1 min (5 min for the first cycle), 54°C for 1 min, and 72°C for 1.5 min (5 min for the last cycle). Each PCR was carried out with a negative control consisting of sterile deionized water in addition to a positive control consisting of DNA extracted from a pure culture of *Escherichia coli* (10 ng/µl). Post-PCR analysis was carried out by electrophoresis of the PCR products on a 1% agarose gel (Amresco) in Tris acetate buffer (0.04 M Tris acetate, 0.001 M EDTA [pH 8.0]) containing ethidium bromide (1 µg/ml). Electrophoresis was carried out for 45 min at 100 V. The amplification products were then visualized and photographed under a UV light transilluminator (AlphaImager, San Leandro, Calif.).

Multiplex PCR with species-specific primers and conserved reverse primer. Multiplex PCR was carried out as previously described (24). The final volume of each PCR mixture was 53.6 µl (comprising 33.6 µl of the master mixture and 20 µl of DNA template). The DNA templates used for multiplex PCR were the amplification products from the first round of PCR using the universal primers 27F and 1492R. A hot-start step was included in this protocol, and AmpliTaq Gold (Applied Biosystems, Foster City, Calif.) was used. The master mixture comprised 10.3 mM Tris-HCl, 51.3 mM KCl (10× PCR buffer II), 2.9 mM MgCl₂, 0.15 µM primer AaF, 0.74 µM primer BfF, 0.49 µM primer PgF, 0.47 µM primer C11R, and 10 U of AmpliTaq Gold. The dNTPs included dATP, dCTP, and dGTP, each at a 0.2 mM concentration, and 600 mM dUTP (all dNTPs were from Promega). The cycling parameters carried out on a Primus thermal cycler consisted of 40 cycles of 95°C for 1 min (10 min for the first cycle), 61°C for 1 min, and 72°C for 5 min (10 min for the last cycle). Each PCR was carried out with a negative control consisting of sterile deionized water, as well as a positive control consisting of genomic DNA extracted from pure cultures of *P. gingivalis*, *A. actinomycetemcomitans*, and *T. forsythensis*, all at a final concentration of 10 ng/µl. Post-PCR analysis was carried out by electrophoresis of the PCR products on a 2.3% agarose gel in Tris-acetate buffer as described earlier. Randomly selected products from relevant groups were excised from the gel, and the DNA was purified using a QIAquick gel extraction kit (Qiagen Ltd., Crawley, United Kingdom). The DNA was then sequenced using a BigDye terminator cycle-sequencing version 2.0 ready-reaction DNA-sequencing kit (AB Biosystems) according to the manufacturer's instructions and analyzed using a 310 Genetic Analyzer (AB Biosystems). The DNA sequences were analyzed online (National Center for Biotechnology Information BLAST [http://www.ncbi.nlm.nih.gov/BLAST]).

Statistical methods. The chi-square test was used to compare the detection percentages of *P. gingivalis*, *A. actinomycetemcomitans*, and *T. forsythensis* in both healthy patients and those with gingivitis, with a *P* value of <0.05 defining significance. Associated odds ratios (ORs) with 95% confidence intervals (CI) were calculated. Logistic regression analysis was performed, and ORs with 95% CI were calculated from its results using the detection percentages for the three pathogens in both healthy patients and those with gingivitis in order to determine the following: (i) if gender and prevalence of the three pathogens were potential risk factors for gingivitis (gingivitis status was the dependent variable, while gender and each of the three pathogens, which were coded as either present or absent, were the explanatory variables) and (ii) if there were differences between the colonization patterns of the three pathogens in male and female children (each of the pathogens was the dependent variable for three logistic-regression analyses, with gender and the remaining two pathogens as the explanatory variables).

The level of statistical significance was set at a *P* value of <0.05. The statistical package used was SPSS version 11.0 (SPSS Inc., Chicago, Ill.).

RESULTS

A total of 118 children were recruited, 64 boys and 54 girls. There were 65 children (36 boys and 29 girls) in group 1 (plaque and no gingivitis) and a further 53 (28 boys and 25 girls) in group 2 (plaque and gingivitis). The mean age was 8.3 (standard deviation, 1.1) years.

The lowest detection limit for *P. gingivalis*, *A. actinomycetemcomitans*, and *T. forsythensis* via PCR using the universal primers 27F and 1492R was 1,000 cells/ml (Fig. 1). The lowest detection limit for all three pathogens after a nested PCR was 10 cells/ml (Fig. 2). No *P. gingivalis*-, *A. actinomycetemcomitans*-, or *T. forsythensis*-specific amplicons were observed with

FIG. 1. Lowest detection levels for *T. forsythensis* after a first round of PCR using the universal 16S rRNA oligonucleotide primers 27F and 1492R. Lane S, molecular size marker (the arrow marks the expected amplicon size of ca. 1,500 bp); lanes 1 to 9, DNA extracted from 10^8 , 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 10^1 , and 1 cells/ml, respectively; lane 10, salmon sperm carrier DNA; lane 11, sterile deionized water.

the negative controls. Specific primers for the three target organisms were used to amplify a range of oral bacteria. No cross-reactions were observed.

The periodontal pathogens *P. gingivalis*, *A. actinomycetemcomitans*, and *T. forsythensis* were scored as positive or negative on the basis of the presence of a clear band of the expected size (Fig. 3). Sequence data from the excised amplicons representing the assumed taxa were analyzed and showed complete agreement. The detection percentages for the three organisms in both healthy patients and those with gingivitis are summarized in Table 1. Chi-square statistic tests revealed that there was a significant difference with respect to the prevalence of *T. forsythensis* in that it was detected more frequently in children with no gingivitis (chi-square test statistic = 4.4; degrees of freedom [df] = 1; $P = 0.03$; 95% CI for difference in detection percentages, 1.6 to 37.0%). There was no statistically significant

difference between those children with and those without gingivitis with respect to the prevalence of *P. gingivalis* (chi-square test statistic = 0.1; df = 1; $P = 0.8$; 95% CI for difference in detection percentages, 16.1 to 20.1%) or *A. actinomycetemcomitans* (chi-square test statistic = 0.1; df = 1; $P = 0.9$; 95% CI for difference in detection percentages, 21.0 to 14.8%).

Logistic regression analysis revealed the following. (i) The odds of a patient having gingivitis were 2.3 times greater in the absence of *T. forsythensis* after adjusting for the gender and prevalence of each of the three pathogens ($P = 0.03$; 95% CI for OR = 1.1 to 4.9). This analysis demonstrated that gingivitis did not appear to be influenced by either gender ($P = 0.9$; OR = 1.1; 95% CI = 0.5 to 2.2) or the presence of *P. gingivalis* ($P > 0.99$; OR = 1.0; 95% CI = 0.5 to 2.2) or *A. actinomycetemcomitans* ($P = 0.5$; OR = 0.8; 95% CI = 0.3 to 1.7). (ii) There was no statistically significant difference in colonization among the three pathogens in male and female children after adjusting for the other pathogens. In particular, the odds of detecting *P. gingivalis* and *A. actinomycetemcomitans* was greater in females than males (OR = 1.48 [95% CI = 0.68 to 3.21], $P = 0.33$, and OR = 1.30 [95% CI = 0.60 to 2.82], $P = 0.51$, respectively). The odds of detecting *T. forsythensis* in females were reduced by 25% compared to those of detecting *T. forsythensis* in males (OR = 0.75 [95% CI = 0.35 to 1.60], $P = 0.45$).

DISCUSSION

The purpose of this work was to estimate the prevalences of *P. gingivalis*, *A. actinomycetemcomitans*, and *T. forsythensis* in plaque associated with the lower first permanent molars before both the onset of puberty and the development of periodontal disease. The lower first permanent molars were sampled because they are the first permanent teeth to erupt and periodontal disease does not usually affect the primary teeth in healthy children. Toothpicks have been used successfully for sampling by other workers (22, 29), and they provide a quick, easy, and effective method of collecting both small and large volumes of supragingival plaque.

Previously designed species-specific primers for the *P. gingivalis*, *A. actinomycetemcomitans*, and *T. forsythensis* 16S rRNA genes (24) can be used in a nested multiplex PCR for the simultaneous detection of these three periodontal pathogens. The specificities of these primers have been confirmed by other workers (24). In addition to primer specificities, amplicons of the three pathogens from plaque were sequenced and confirmed to match those contained within the National Center for Biotechnology Information BLAST database.

Genomic DNA belonging to the oral gram-negative bacterium *C. ochracea* was added to the reaction mixtures for the detection limit analysis to mimic background DNA arising from other organisms and host cells that would have been present in the plaque samples. The genomic DNA from *C. ochracea* in the limit-of-detection analysis did not appear to interfere with the PCR. There was a potential risk when preparing genomic DNA extracts for the limit-of-detection analysis that the DNAs from cultures with low cell numbers would remain unprecipitated. The use of carrier DNA in a sufficient concentration can be used to encourage the recovery of small amounts of DNA (15). The use of eukaryotic carrier DNA,

FIG. 2. Lowest detection levels for *T. forsythensis* after nested PCR using the species-specific primer BfF and the conserved reverse primer C11R. Lane S, molecular size marker (the expected amplicon size for *T. forsythensis* was ca. 745 bp [marked by the symbol Tf]); lanes 1 to 9, 16S ribosomal DNA (rDNA) from 10^8 , 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 10^1 , and 1 cells/ml, respectively; lane 10, positive control comprising 16S rDNA (10 ng/ μ l) from the *T. forsythensis* type strain ATCC 43037; lane 11, positive control comprising 16S rDNA (10/ μ l) from *C. ochracea* ATCC 27872 (the expected amplicon size for *C. ochracea* was ca. 205 bp [marked by the symbol Co]); lane 12, negative control comprising sterile deionized water and 16S rDNA (10 ng/ μ l) from *C. ochracea*; lane 13, negative control comprising sterile deionized water used in the first round of PCR; lane 14, negative control comprising salmon sperm DNA; lane 15, negative control comprising salmon sperm DNA used in the first round of PCR.

FIG. 3. Multiple DNA bands observed for the three pathogens *P. gingivalis*, *A. actinomycetemcomitans*, and *T. forsythensis* after multiplex PCR. Lane S, molecular size marker (the expected amplicon sizes for the three pathogens are 197, 360, and 745 bp [marked by the symbols Pg, Aa, and Tf]). Lanes 1 to 6 comprise 16S (rDNAs) amplified from dental plaque. Lane 1, amplicons of the expected sizes for all three pathogens can be observed; lane 2, an amplicon of the expected size for *P. gingivalis* can be clearly observed, as well as a weak amplicon of the expected size for *T. forsythensis*; lane 3, amplicons of the expected sizes for all three pathogens can be observed; lane 4, amplicons of the expected sizes for *P. gingivalis* and *A. actinomycetemcomitans* can be observed; lane 5, amplicons of the expected sizes for all three pathogens can be observed; lane 6, positive control comprising 16S rDNAs (10 ng/ μ l) from the type strains *P. gingivalis* NCTC 11834, *A. actinomycetemcomitans* NCTC 9710, and *T. forsythensis* ATCC 43037; lane 7, negative control comprising sterile deionized water; lane 8, negative control comprising sterile deionized water used in the first round of PCR.

such as salmon sperm DNA, would thus encourage the genomic DNA extracted from bacteria with low cell numbers to become precipitated. No amplicons specific for *P. gingivalis*, *A. actinomycetemcomitans*, and *T. forsythensis* were observed when salmon sperm DNA was used as a second negative control. The nested multiplex PCR detection methodology used in the present study has a limit of detection of 10 bacterial cells for all three primers and is similar to those reported by earlier workers (24). Nested multiplex PCR is therefore much more sensitive for bacterial detection than other molecular approaches, such as DNA hybridization assays, where the lowest detection reported has been on the order of 10^2 to 10^3 cells (3). For the purpose of the present study, failure to detect a band was interpreted as the bacteria being either absent or present in numbers lower than 10 cells. Any weak or faint DNA bands (concentrations under 1 μ g/ml) on gel photographs were regarded as such or even as possible DNA carryover from other

wells. PCRs were repeated for a number of plaque samples either to confirm the presence of a weak band or as a quality control measure. Weak bands observed after >1 PCR were considered to be positive for the organism of interest. An example of this can be observed in Fig. 3, lane 2, where a weak band of the expected size for *T. forsythensis* was considered to be negative (when replicated).

T. forsythensis was observed significantly more frequently in children without gingivitis. Although this is statistically significant, the clinical significance of the finding has yet to be determined. There was, however, no significant difference between the prevalences of *P. gingivalis* or *A. actinomycetemcomitans* in the two groups of children. Logistic regression analysis revealed that gender was not a potential risk factor for the development of gingivitis. It also demonstrated that the colonization of plaque by the three pathogens was not influenced by gender.

Although plaque was sampled from children from a variety of ethnic backgrounds, correlations between ethnicity and pathogen colonization were beyond the aims of the present study. Further studies specifically addressing ethnicity and/or socioeconomic groups need to be carried out.

Lower prevalences for the periodontal pathogens *P. gingivalis* (8.3%), *A. actinomycetemcomitans* (2.8%), and *T. forsythensis* (2.7%) in the permanent dentition of children aged 7 to 8 years have been reported in a study involving cultivation of the organisms (8). Other workers have failed to detect the three pathogens in young children by culture (6). In the latter study, plaque was sampled from children aged 0 to 2.5 years (the ages of children in the present study were 5 to 9 years), 10-fold serially diluted, and then streaked onto selective media. Recent culture studies have revealed that *P. gingivalis*, *A. actinomycetemcomitans*, and *T. forsythensis* belong to a group of oral pathogens that are significant markers for destructive periodontal disease in adult subjects (25). *P. gingivalis* and *T. forsythensis* were the strongest bacterial markers for disease and were infrequently cultured from subjects without periodontal bone loss. Since the samples in all of these studies were serially diluted and then cultured, the detection limit for these taxa would have been very high and target bacteria could have been diluted out. Sites of periodontal bone loss may have increased numbers of the three pathogens, allowing more frequent detection by culture after serial dilutions. Other investigators (9) using the same species-specific primer for *P. gingivalis* used in the present study failed to detect the pathogen in any of their subjects. Methods analogous to those of the present study were used, with the exception of the use of a single round of PCR (with a stated detection limit of 100 cells). Therefore, it is conceivable that these three pathogens may be present in

TABLE 1. Detection of the three dental pathogens studied

Group ^a	No. of subjects	<i>P. gingivalis</i>		<i>A. actinomycetemcomitans</i>		<i>T. forsythensis</i>	
		No. of positive subjects	% Detection (95% CI)	No. of positive subjects	% Detection (95% CI)	No. of positive subjects	% Detection (95% CI)
1	65	32	49.2 (33.6–61.9)	36	55.4 (42.5–67.7)	42	64.6 (51.8–76.1)
2	53	25	47.2 (33.3–61.4)	31	58.5 (44.1–71.9)	24	45.3 (31.6–59.6)

^a Group 1, no gingivitis; group 2, with gingivitis.

plaque but not be detected as a result of their being present in low numbers.

Although in the present study the plaque was obtained from young children (5 to 9 years old), there is evidence to suggest that colonization of *P. gingivalis*, *A. actinomycetemcomitans*, and *T. forsythensis* can occur from a very young age, indeed, as young as 1.5 years (21). Other investigators have identified *P. gingivalis* and *A. actinomycetemcomitans* in the plaque of young children by using PCR analysis (10, 13). *P. gingivalis* was detected in 40 to 50% of children ranging from 0 to 2 years of age, but the greatest prevalence (60%) was observed in teenagers aged 13 to 14 years (13). *A. actinomycetemcomitans* was detected in 25 to 50% of children ranging from 0 to 3 years of age, with the greatest prevalence (58%) in children aged between 5 and 9 years (10). The use of PCR techniques for the detection of these three pathogens is a more sensitive method than culturing because DNA from bacteria with low cell numbers can be amplified and identified.

The presence of these three principal periodontal pathogens in both health and disease would suggest that other factors, such as pathogen numbers, might be responsible for causing disease. This substantiates the principles of the ecological plaque hypothesis (12), which proposes that organisms associated with disease can be present at healthy sites but at levels too low to be clinically significant. It is, however, evident that these pathogens are present in periodontally healthy children and that colonization may occur at a very young age (21). This could also further support the claim that these organisms may truly be endogenous in the oral cavity. What is still uncertain is whether the early colonization by these three pathogens in children can be regarded as a risk factor for future development of periodontitis.

The high prevalence of *T. forsythensis* in children with no gingivitis was unexpected. Previous workers (14) had failed to detect this pathogen in 2- to 12-year-old children with no gingivitis after one round of PCR (detection limits not stated). However, other investigators (21) have detected *T. forsythensis* in the plaque of 18% of very young children (aged 19 to 36 months) by using the checkerboard hybridization assay (17) with a detection limit of $>10^4$ cells. The increased sensitivity for detecting these periodontal pathogens through the use of nested multiplex PCR may help to explain the higher detection frequency observed in the present study.

Further work is needed to analyze the significance of finding a greater prevalence of *T. forsythensis* in children with no gingivitis than in children with gingivitis as observed in the present study. A quantitative assay employing, for example, real-time PCR targeting these three pathogens in sites with and without gingivitis may demonstrate differences in the actual numbers. Using this technique, clinical signs and symptoms could be correlated with actual numbers of each of the pathogens. In conclusion, the results of this study have shown that the three pathogens can be detected in the plaque of children with and without gingivitis and specifically that *T. forsythensis* is associated with dental plaque found at sites without gingivitis.

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