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Gram-positive anaerobic bacilli in human periodontal disease

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Objective: The uncertain taxonomy of oral anaerobic gram-positive bacilli and their generally slow growing nature has limited the understanding of their role in periodontal disease. The current objective was to design and use species-specific oligonucleotide probes to investigate the relationship of selected gram-positive anaerobic bacilli to periodontal disease.

Methods: Plaque and clinical measurements were collected from 40 patients with periodontitis and from 40 matched controls. Oligonucleotide probes were designed for *Bulleidia extructa*, *Eubacterium nodatum*, *Mogibacterium timidum* and *Slackia exigua* and used to probe nucleic acids extracted from the samples with a chemiluminescent detection method. Species were quantified as absent or present at low (approximately 10^3–10^4 cells), medium (approximately 10^4–10^5 cells) or high levels (approximately 10^5–10^6 cells).

Results: *M. timidum* and *B. extructa* were detected in only three and four samples, respectively. The level of both *E. nodatum and S. exigua* was significantly higher in deep than shallow pockets (Wilcoxon, $p < 0.001$). The level of *E. nodatum*, but not *S. exigua*, was higher in patients than matched controls (Mann–Whitney U, $p < 0.03$). Using an ordered logistic regression model, the probing depth of the sampled sites had the greatest influence on the level of both species and significant variations occurred between individuals. Bleeding also influenced the levels of both species, with supragingival plaque influencing *S. exigua*.

Conclusion: Both *E. nodatum and S. exigua* were associated with clinical indicators of periodontal disease.

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Human periodontal disease results from the interaction between plaque bacteria and a susceptible host. Historically, bacteria in dental plaque have been investigated using cultural methods, which discriminate against finding slow-growing or unculturable species. Many cultural studies have identified a predominantly gram-negative microflora associated with periodontal disease. Certain gram-negative species, e.g. *Porphyromonas gingivalis,* which are readily detectable by cultural methods, are undoubtedly associated with the disease (1, 2) but proof of their role as causative pathogens remains elusive. However, studies describing the total cultivable flora also suggested a significant role of certain gram-positive bacteria in periodontitis, particularly those species formerly assigned to the genus *Eubacterium* (3, 4). In addition, serum antibody responses to species of *Eubacterium* could distinguish serum from periodontitis patients from the serum of matched healthy controls (5). Culture-independent molecular analysis of plaque from severe periodontitis suggested that cultural analysis severely underestimates the numbers of gram-positive bacteria, which can represent over 50% of the anaerobic microflora (6).

Many of the species formerly assigned to the genus *Eubacterium* are difficult to grow and distinguish from each other by biochemical tests, making it difficult to determine their role in disease. In addition, because it is

defined by default the genus has become a heterogeneous collection of taxa. Therefore, for those bacteria distinctly different from the type species *Eubacterium limosum*, based on biochemical and molecular characteristics, recent taxonomic studies have proposed new genera to accommodate some species formerly assigned to the genus *Eubacterium*, new species to accommodate novel taxa and reassignment of other species to the appropriate genus (7–10).

The aim of this work was to develop a culture-independent method of detecting *Bulleidia extructa*, *Eubacterium nodatum*, *Mogibacterium timidum* and *Slackia exigua* and to investigate the relationship of these four species of gram-positive bacilli with human periodontal disease.

Material and methods

Study population

Forty patients with chronic periodontitis attending the Dental Teaching Hospital of Bart's and the London School of Medicine and Dentistry were included in this study. Patients were invited to participate in the study based on a previous periodontal assessment and all patients had two or more sites with loss of attachment greater than 6 mm. In addition, patients were medically healthy, aged at least 25 years and had neither antibiotics nor any form of periodontal therapy in the 6 months prior to participating in this study. Forty age and gender-matched controls without periodontitis were also included in this study. Ethical approval for the study was obtained from the East London and City Research Ethics Committee.

Clinical measurements

At sampled sites supragingival plaque was scored as clearly visible, detectable only with a probe or absent and was removed before subgingival plaque was collected. Sites were probed manually and bleeding after probing scored as immediate (score = 2), within 30 s (score = 1) or absent (score = 0). Recession was recorded if present. To determine the general level of oral hygiene and marginal gingival health, plaque and gingival bleeding indices were recorded on buccal and lingual surfaces of the six 'Ramfjord teeth'. One examiner made all clinical measurements and collected all samples.

Subgingival plaque samples

Four subgingival plaque samples were collected from each patient, two from inflamed sites with probing depths of at least 6 mm and two from relatively healthy sites with probing depths of 4 mm or less and little or no attachment loss. Subgingival plaque samples were taken from two sites with shallow probing depths in each of the matched controls. Supragingival plaque was carefully removed and a subgingival plaque sample was collected using a sterile curette. The plaque sample was suspended in 500 μl storage buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at −70°C.

Oligonucleotide probes

Regions of the 16S rRNA gene specific for each species were identified by visual inspection of alignments with related species obtained with the Clustal W programme. Multiple potential primers were selected for each target species and checked for accessibility to the 16S rRNA molecule by comparison with the secondary structure map described by Fuchs *et al.* (11). The probes, labelled at the 3′ end with digoxigenin, were supplied by MWG Biotech (Milton Keynes, UK). Probes predicted to give high intensity signal were then tested for specificity by slot blot hybridization against 46 species representing both oral and non-oral taxa from different phyla (Table 1). To determine their sensitivity, each probe was hybridized with between 10 and 10^6 cells of the target species, in 10-fold steps, as determined by plate colony counts. The probes chosen for use, on the basis of good sensitivity and lack of cross-reaction with other taxa, were as follows: *B. extructa* – 5′-GAT GCCTCTCTTCTACCTAT-3′, *E. nodatum* – 5′-TTCTTTAAGTGATTT CTCGCTC-3′, *M. timidum* – 5′-CC GCCGCTGTCCGCTTGG-3′ and *S. exigua* – 5′-CCGTCCCCTCCCGC CAAG-3′. A 'universal' probe Eub338 (GCTGCCTCCCGTAGGAGT) (12) was used to estimate the total microbial 16S rRNA content in the plaque samples. A 'nonsense' probe (Eub338 reversed) (TGAGGATGCCCTCCGT CG) was used to detect non-specific binding to the nucleic acids.

Nucleic acid extraction

For each patient, the plaque samples from deep pockets were pooled, as were those from shallow pockets in both patients and controls. Pilot work had indicated that detection of the target species in subgingival plaque samples was optimized if plaque was pooled from more than one site. Nucleic acid was extracted from the samples using a modification of a method optimized for gram-positive bacteria (13). Samples were centrifuged and the pellet resuspended in 500 μl of buffer containing 0.1 M sodium chloride, 10 mM Tris-HCl, 1 mM EDTA and 1% Triton-X (TEST) with 10 mg/ml lysozyme, vortexed and incubated at 37°C for 20 min. Proteinase K (1 mg/ml) and 100 μl 20% sarkosyl were added and incubated at 37°C for a further 40 min. After extraction with an equal volume of phenol/chloroform/isoamyl alcohol (25 : 24 : 1): total nucleic acids were precipitated with ethanol and suspended in 30 μl of sterile distilled water. The 46 species used to determine the specificity of the probes were suspended directly into the TEST buffer and the same extraction procedure followed.

Slot blot hybridization

RNA in the samples was denatured by the addition of 90 μl 2% gluteraldehyde and then diluted with 510 μl of 6 × saline-sodium citrate buffer, pH 7 (0.09 M sodium citrate and 0.9 M sodium chloride) with poly A (total volume 630 μl). One hundred microlitres of sample was blotted onto nylon membranes (Hybond^{TM}-N+, Amersham Biosciences UK Ltd, Chalfont St. Giles, UK) with an 8 × 12 slot blot

Table 1. Bacterial strains used for the validation of species-specific probes

Actinobacillus actinomycetemcomitans ATCC 43718
Atopobium rimae ATCC 49626
Bacteroides fragilis ATCC 25285
Bulleidia extructa DSM 13220
Campylobacter rectus ATCC 33238
Clostridium perfringens W1678
Cryptobacterium curtum D125H
Eggerthella lenta NCTC 11813
Erysipelothrix rhusiopathiae NCTC 8163
Escherichia coli NCTC 10418
Eubacterium brachy ATCC 33089
Eubacterium limosum CCUG 16793
Eubacterium minutum ATCC 700079
Eubacterium nodatum ATCC 33099
Eubacterium saphenum ATCC 49989
Eubacterium sulci ATCC 35585
Eubacterium yurii ATCC 43714
Filifactor alocis ATCC 35896
Filifactor villosus DSM 1645
Fusobacterium nucleatum ATCC 25586
Holdemania filiformis ATCC 51649
Lactobacillus casei W2005
Mogibacterium timidum ATCC 33093
Peptostreptococcus anaerobius NCTC 11460
Peptostreptococcus micros ATCC 33270
Porphyromonas asaccharolytica ATCC 25260
Porphyromonas endodontalis ATCC 35406
Porphyromonas levii NCTC 11028
Prevotella bivia NCTC 11156
Prevotella denticola ATCC 35308
Prevotella intermedia ATCC 25611
Prevotella loescheii NCTC 11321
Prevotella melaninogenica ATCC 25845
Prevotella nigrescens NCTC 9336
Prevotella oralis NCTC 11459
Prevotella oris ATCC 33573
Prevotella oulorum ATCC 43324
Prevotella ruminicola ATCC 19189
Rikenella microfusus NCTC 11190
Shuttleworthia satelles DSM 14600
Slackia exigua ATCC 700122
Slackia heliotrinireducens ATCC 29202
Solobacterium moorei JCM 10645
Staphylococcus aureus NCTC 6571
Streptococcus mutans NCTC 10832
Tannerella forsythensis ATCC 43037

hybridization manifold (Jencons (Scientific) Ltd, Leighton Buzzard, UK) for each of the six probes. In addition, three 10-fold dilutions were made of the samples and 100 μl of each dilution loaded onto membranes for the universal and nonsense probes. Membranes were baked at 80°C for 2 h. Non-isotopic hybridization and detection of the digoxigenin-labelled probes with the chemiluminescent alkaline substrate CSPD^R (Roche DIG System for Filter Hybridization; Roche Diagnostics Ltd, Lewes, UK) was performed according to the manufacturer's instructions. Briefly, membranes were prehybridized for 2 h at 48°C in a hybridization oven (Biometra, Gottingen, Germany) with 20 ml buffer [5 × saline-sodium citrate buffer, 0.1% (w/v) *N*-lauroyl-sarcosine, 0.2% (w/v) sodium dodecyl sulphate, 1% Blocking Reagent]. Membranes were then hybridized overnight at 48°C in 20 ml fresh buffer containing 400 pmol of probe. Post-hybridization washes were performed with a 2 × saline-sodium citrate buffer wash solution at room temperature and then a 0.5 × saline-sodium citrate buffer wash solution at 48°C for the universal and nonsense probes, 56°C for *B. extructa,* 57°C for *E. nodatum and* 58°C for *M. timidum* and *S. exigua* probes. The temperatures for the 0.5 × saline-sodium citrate buffer stringency washes were determined experimentally for each probe to optimize probe specificity and sensitivity. Following incubation with Anti-Digoxigenin-alkaline phosphatase and then CSPD^R, chemiluminescence was visualized by exposure to X-ray film. In order to estimate the numbers of cells in the sample, known dilutions of the target species were processed with each blot. Hybridization signals were then quantified by direct visual comparison with these controls. The target species was considered to be present in plaque at a low level if the signal corresponded with or exceeded the signal from approximately 10^3 cells of the target species but was below the level of the signal from 10^4 cells, at a medium level if it corresponded with the signal from approximately 10^4 cells but was below that of 10^5 cells and at a high level if it exceeded the signal from approximately 10^5 cells.

Statistical analyses

The mean plaque and gingival bleeding index was calculated for each patient. Since plaque samples had been pooled for the microbial analysis, the mean per patient of the clinical measurements at sampled diseased and sampled healthy sites were used in the analyses. Mann–Whitney *U*-tests were used to compare the levels of *E. nodatum*, *S. exigua* and total bacteria, the bleeding and plaque indices, and the clinical variables from sampled sites in patients and controls. Wilcoxon sign rank tests were used to compare the microbial and clinical variables from the shallow and deep pockets in the patients with periodontitis. An ordered logistic regression analysis model was used to test the influence of the patients' age, gender, clinical variables and the size of the plaque sample indicated by the total level of bacteria on the levels of *E. nodatum* and *S. exigua*. The patient

was the primary sampling unit in the regression model.

Results

All four probes were specific for their target species with no hybridization detected at the specified wash temperatures when tested against the 46 reference strains. The *B. extructa*, *E. nodatum* and *S. exigua* probes were able to detect 10^3–10^4 cells. The sensitivity of the *M. timidum* probe was 10^4–10^5 cells. An example of a blot showing detection of *E. nodatum* is illustrated in Fig. 1.

E. nodatum was detected in 29 of the 40 samples from deep pockets, 16 of the 40 samples from shallow pockets in patients with periodontitis and only seven samples from control subjects (Table 2). There were significantly higher levels of *E. nodatum* detected in deep than in shallow pockets in patients (Wilcoxon sign rank test $Z = -4.5$, $p < 0.001$) and also higher levels of *E. nodatum* detected in the shallow pockets of patients than in control subjects (Mann–Whitney *U*-test $Z = -2.26$, $p < 0.03$).

S. exigua was found more frequently than *E. nodatum* and was detected in 36 pooled samples from deep pockets, 21 samples from shallow pockets in patients and 17 samples from control subjects (Table 2). The level of *S. exigua* was significantly higher in samples from deep pockets than shallow pockets in patients (Wilcoxon sign rank test $Z = -4.6$, $p < 0.001$), but there were no significant differences found in the shallow pockets of patients and control subjects.

The size of the plaque samples indicated by the intensity of signal with the universal primer (Table 2) was significantly greater from deep than shallow pockets in patients (Wilcoxon sign rank test $Z = -4.02$, $p < 0.001$) and from the shallow pockets of patients than controls (Mann–Whitney *U*-test $Z = -2.78$, $p < 0.006$).

B. extructa was detected in three shallow pockets and one deep pocket in patients. *M. timidum* was detected in two control subjects and one shallow pocket in a patient. The sensitivity of the assay to detect *M. timidum* was 10^4–10^5 cells and this may have accounted for the lower detection of this species in plaque. Because of their low rate of detection, the data for these species have therefore not been subjected to statistical analysis.

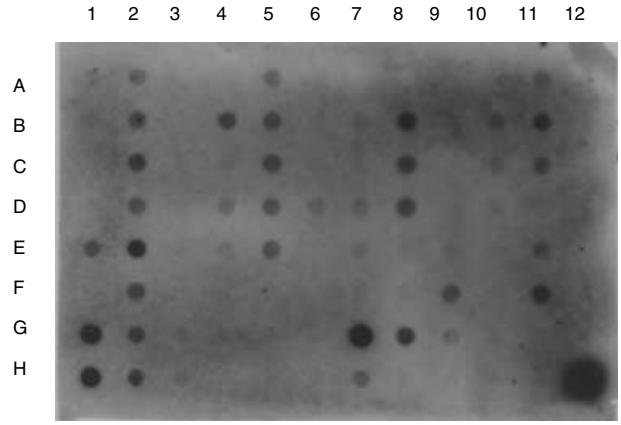

Fig. 1. Slot blot hybridization detection of *Eubacterium nodatum* in plaque from periodontitis patients and control subjects. Rows A–F, columns 1, 4, 7, 10: shallow pockets, patients; rows A–F, columns 2, 5, 8, 11: deep pockets, patients; rows A–F, columns 3, 6, 9, 12: shallow pockets, controls. G1–6, G7–12, H1–6 – *E. nodatum* control, 10^6–10 cells in ten-fold dilutions. H7–12 – internal hybridisation controls.

Table 2. The level of microorganisms in plaque samples from patients and control subjects

Level of microorganism	Patients with periodontitis		Controls
	Deep pockets	Shallow pockets	Shallow pockets
Eubacterium nodatum			
Not detected	11	24	33
Low	5	12	6
Medium	20	4	1
High	4	0	0
Slackia exigua			
Not detected	4	19	23
Low	13	18	13
Medium	23	3	4
Total 16S rRNA			
Low	2	9	20
Medium	21	26	19
High	17	5	1

Patients and clinical parameters

There was no significant difference in the mean age of the patients (45.3 years, SD = 9.14) and controls (44.9 years, SD = 9.20). The level of oral hygiene indicated by the plaque index (Fig. 2b) was significantly better in the controls than the patients (Mann–Whitney *U*-test $Z = -5.37$, $p < 0.0001$) and the marginal gingival health (Fig. 2a) of controls was also better than that of the patients (Mann–Whitney *U*-test $Z = -3.67$, $p < 0.001$).

Sampled sites

The mean pocket depth of sites with periodontitis selected for microbial sampling was 7.24 mm (SD = 0.82). The shallow pockets in the patients had a mean probing depth of 2.79 mm (SD = 0.37) and sampled sites in the control subjects had a mean probing depth of 2.43 (SD = 0.45).

All sampled sites had supragingival plaque detected. There was a statistically significant difference in the

supragingival plaque index (Table 3) at deep and shallow sites sampled in the patients (Wilcoxon signed rank test $Z = -3.76$, $p < 0.01$) and between shallow sites in the patients and control subjects (Mann–Whitney *U*-test $Z = -2.63$, $p < 0.01$). The level of inflammation indicated by bleeding after probing (Table 3) was significantly more severe in the deep sites with periodontitis compared with the relatively healthy shallow sites in the patients (Wilcoxon signed rank test $Z = -4.15$, $p < 0.0001$). There was no significant difference in the bleeding index at shallow sites in patients and control subjects.

In view of the variations in the levels of both *E. nodatum* and *S. exigua* in sites with different clinical conditions, an ordered logistic regression analysis was conducted to examine further the factors and clinical parameters which might account for the level of the microorganisms. In the ordered logistic regression model the influence of age and gender together with the clinical variables were investigated for their effect on *E. nodatum* and *S. exigua*. Since there were significant variations in the quantity of plaque sampled from different sites and since this would be likely to influence the detection and quantification of specific species in those samples, the size of the plaque sample estimated by the *Bacteria*-specific probe was also included in the regression model.

The factors most significantly influencing the level of *E. nodatum* were the probing depth of the sampled site and individual variation between patients (Table 4). Whether the patient had periodontitis or were a healthy control was also an explanatory factor as was the level of gingival inflammation indicated by the bleeding index. The size of the plaque sample showed a trend towards a significant influence on levels of *E. nodatum* ($p < 0.06$). Age, gender and supra-gingival plaque levels had no significant effect on *E. nodatum* in this model.

The probing depth of the sampled site had a significant effect on the level of *S. exigua* as did the bleeding index and level of supragingival plaque (Table 5). The influence of the

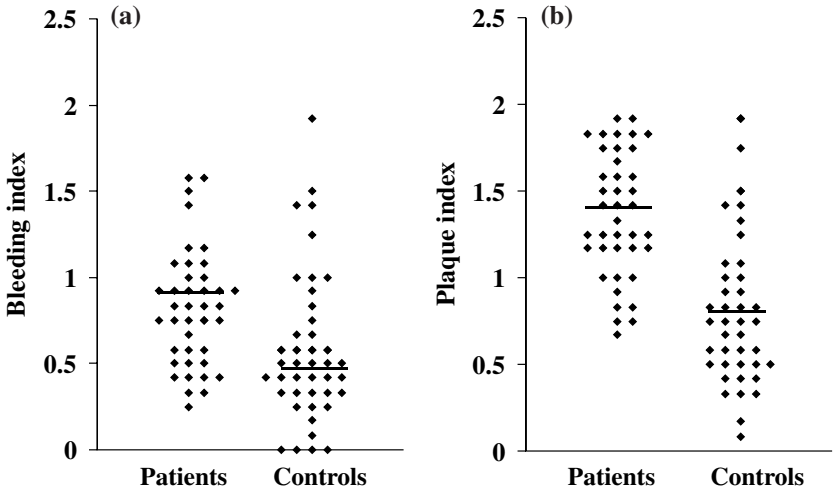

Fig. 2. (a) Gingival inflammation (bleeding index) and (b) supragingival plaque (plaque index) in patients with periodontitis and matched controls. Solid line indicates the median.

Table 3. Supragingival plaque and bleeding after probing at sampled sites

Clinical variable at sampled site	Number of sites		
	Patients		Controls
	Deep	Shallow	Shallow
Supragingival plaque			
0	0	0	0
1	7	22	44
2	73	58	36
Bleeding after probing			
0	2	8	10
1	18	42	52
2	60	30	18

Table 4. Ordered logisitic regression model for high, medium, low or undetectable levels of *Eubacterium nodatum* in patients with periodontitis and control subjects

Explanatory variable	Coefficient	s.e.	*t*	95% confidence interval		*p*
Individual patient	0.032	0.012	2.71	0.008	0.055	0.008
Patient or Control	0.781	0.383	2.04	0.019	1.543	0.045
Bleeding index	1.480	0.710	2.09	0.067	2.895	0.040
Probing depth	0.444	0.114	3.90	0.217	0.671	0.000
Total 16S rRNA	0.760	0.394	1.93	−0.025	1.545	0.057

Table 5. Ordered logisitic regression model for medium, low or undetectable levels of *Slackia exigua* in patients with periodontitis and control subjects

Explanatory variable	Coefficient	s.e.	*t*	95% confidence interval		*p*
Individual patient	0.016	0.008	2.10	0.0009	0.0315	0.039
Supragingival plaque	1.298	0.574	2.26	0.155	2.441	0.027
Bleeding index	1.212	0.592	2.05	0.035	2.390	0.044
Probing depth	0.489	0.127	3.86	0.237	0.741	0.000
Total 16S rRNA	0.377	0.366	1.03	− 0.352	1.107	0.306

individual patient reached statistical significance, but the regression model seems to confirm the finding of the simple Mann–Whitney *U*-tests that there was no significant effect dependent on whether the sample had been collected from a patient or control subject.

Discussion

The problems associated with culturing and isolating four species of anaerobic non-sporing gram-positive bacilli from plaque samples were overcome in this study by designing and using species-specific digoxigenin-labelled oligonucleotide probes. It was also possible to quantify the species within plaque as present at low, medium or high levels. Known numbers of cultured cells, established by colony counts of the species in pure culture, were processed with the plaque samples and indicated that a 'low' level corresponded with the hybridization signal generated by between 10^3 and 10^4 cells, the 'medium' level corresponded with the signal from 10^4 to 10^5 cells and a 'high' level with signal from 10^5 to 10^6 cells. The level of sensitivity using the probes designed in this study compares favourably with that described by other workers. Non-radioactive methods similar to those employed here have been reported as being able to detect 10^4–10^5 cells (14), whereas the use of ^{32}P allows detection of 10^2–10^3 cells (15). Three of the probes used here showed sensitivity between these values. Greater sensitivity would be desirable and a method of accurately quantifying very low numbers of specific species would be useful to establish if there is a threshold level above which particular species become clinically significant. Currently, unless there is a detection threshold, in an open ecosystem such as the mouth, every organism might be detected at every site.

Whole genomic DNA probes have been proposed as a sensitive method of microbial identification and have been used in a 'checkerboard' format to detect a range of periodontal microorganisms in a single plaque sample (16, 17). However, the technique relies on hybridizing DNA from all test microorganisms at the same temperature, which may lead to cross-reaction between DNA of different species. In addition, the species specificity of probes can only be validated against the DNA of culturable microorganisms. It is now understood that the oral flora is far more complex than was previously believed, with over 600 species described, of which fewer than half can currently be cultured (18).

Oligonucleotide probes targeting 16S rRNA can be designed with species specificity by inspection of aligned sequences of taxa closely related to the target organism, both culturable and non-culturable. However, this improved specificity is offset by their low sensitivity compared to whole genomic probes. One way to overcome this is to incorporate a PCR step into the procedure to provide considerably more target for the probes. A reverse-capture PCR method in which the target DNA is first amplified with *Bacteria*-specific primers has been used in order to improve the sensitivity of detection with oligonucleotide probes (19). However, it has been recognized that primer bias can have a major influence on the proportions of PCR product obtained in mixed-template PCRs. For example, in a combined culture/molecular study of the endodontic microflora it was found that PCR underestimated the number of members of the phylum *Actinobacteria* (high G + C gram-positive) compared to culture (20).

The control subjects were selected because they did not have significant destructive periodontal disease, but they also differed from the patients as their marginal gingivae were less inflamed and they had significantly less supragingival plaque. Both the severity of inflammation and the level of supragingival plaque could influence the nature of the subgingival habit creating suitable conditions for different subgingival species. Sampling both deep pockets and relatively healthy shallow pockets within the patients provided an additional control, which standardized for individual variations in systemic and genetic factors.

It is not easy to overcome the difficulties of comparing the bacteria within two different types of habitat in the patient group or the difficulties of comparing bacteria from the clinically more similar, healthier habitats in different individuals. However, simple comparisons of *E. nodatum* at shallow sites in periodontitis patients and healthy controls and at deep and shallow sites in the patient group suggested that *E. nodatum* was associated with periodontal disease as described previously (4, 19, 21–23). The ordered regression modelling helped to clarify that the level of *E. nodatum* was significantly higher in the patient group than healthier controls, but that there was also a highly significant effect of the individual on *E. nodatum*. Variations in each host's immune and inflammatory responses might account for this. In addition, there will also be differences in the indigenous microflora of individuals with which *E. nodatum* might interact.

The depth of sampled pockets had a particularly significant influence on the level of *E. nodatum* and this was still significant when the greater quantity of plaque sampled from deep pockets was taken into account in the regression model. This helped to exclude the possibility that the deeper pocket provided a larger plaque sample with the obviously increased likelihood of detecting more of a specific species, and suggests that it may have been the quality of the habitat provided by the deep pocket that influenced *E. nodatum*. The level of gingival inflammation also had a significant influence on *E. nodatum* and this might be explained because inflammation results in an increased flow of gingival crevicular fluid containing a range of nutrients for the bacteria in the crevice or pocket. Whether this had a direct effect on *E. nodatum* or an indirect effect mediated by other members of the microflora is unknown.

S. exigua has been found in dentoalveolar and endodontic infections (24, 25). Its relationship with periodontal disease is less clear, although it contributed to the asaccharolytic eubacteria found in plaque from periodontitis (26), and serum antibodies

against *S. exigua* were detectable in some periodontitis patients (27). In the current work *S. exigua* was found more frequently than *E. nodatum*, particularly in the relatively healthy control group. The regression model suggests that there was a significant effect of the individual on *S. exigua*, but seems to confirm that there were no significant differences which depended on whether the samples came from patients or controls. However, the level of *S. exigua* detected was significantly influenced by the pocket depth and level of inflammation. *S. exigua* was also significantly influenced by the amount of supragingival plaque at sampled sites and this might suggest interactions between *S. exigua* and other members of the microflora not examined in this work. Analysis of supragingival plaque for the test species was not undertaken in this study but may add interest to future work, particularly if *S. exigua* can be influenced by supragingival plaque. In this study subgingival plaque was analysed because of its relevance to destructive periodontal disease and also because the target species were strictly anaerobic.

In conclusion, *E. nodatum* and *S. exigua* were clearly associated in this case control cross-sectional study with clinical indicators of periodontal disease. As with other studies, it is not possible to conclude that these species are causally related to the disease. However, it does confirm the need to investigate further the role of gram-positive species in the disease process and to include such species together with the well-characterized gram-negative species, e.g. *P. gingivalis,* in future work. Large studies employing molecular methods of quantification, such as those used in the current work, would be particularly beneficial. However, further work to improve the sensitivity of the technique while maintaining the high specificity of the oligonucleotide probes would be beneficial. The association, particularly of *E. nodatum* with disease also suggests a need for cultural studies to help characterize its virulence factors as these might provide targets for novel therapy.

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