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Enumeration of *Porphyromonas gingivalis*, *Prevotella intermedia* and *Actinobacillus actinomycetemcomitans* in subgingival plaque samples by a quantitative-competitive PCR method

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Porphyromonas gingivalis, *Prevotella intermedia* and *Actinobacillus actinomycetemcomitans* are believed to play an important role in adult periodontitis, but the significance of their relative numbers and progress of the disease is still unclear. Traditional quantitative methods are generally time-consuming and inaccurate. The aim of this study was to develop a sensitive, quantitative PCR technique that would be useful for enumerating *P. gingivalis*, *Pr. intermedia* and *A. actinomycetemcomitans* in subgingival plaque samples from subjects with adult periodontitis. Primers to the following genes were employed: the fimbrial gene (*fimA*) of *P. gingivalis*, the 16S rRNA gene of *Pr. intermedia* and the leukotoxin-A (*lktA*) gene of *A. actinomycetemcomitans*. Competitive templates were constructed either by sequence deletion between primer binding sites or by annealing of the primer binding sites to an appropriate DNA core so as to yield products of a different size from that obtained with the target template. Co-amplification of target and competitive templates yielded products of expected size and non-specific recognition by the primers was not found. The sensitivity of the designed primers was 100 cells of *P. gingivalis*, 100 cells of *Pr. intermedia* and 10 cells of *A. actinomycetemcomitans*. The three species were found in subgingival plaque samples collected from both healthy and diseased sites by the quantitative-competitive (QC)-PCR method and the technique was more sensitive than cultural methods. For determining the proportions of each of the three periodontopathogens, the total number of bacteria in the samples was enumerated by quantitative-PCR with 16S rRNA universal primers (27f and 342r). The findings indicate that QC-PCR is a useful method for enumerating bacteria in clinical oral specimens and the technique could play a role in the investigation of disease progression.

Introduction

Chronic inflammatory periodontal diseases are some of the most widespread bacterial diseases affecting mankind and adult periodontitis is the most frequent type. More than 200 species of micro-organisms colonise the oral cavity, but only a few of these are thought to be periodontal pathogens [1]. Among the subgingival bacterial species identified so far, *Porphyromonas gingivalis*, *Prevotella intermedia* and *Actinobacillus actinomycetemcomitans* have been associated

with progressive adult periodontitis [2]. *A. actinomycetemcomitans* is infrequently found in periodontally healthy individuals [3], whereas *Pr. intermedia* has been found in healthy subjects and is more frequent in patients with periodontal diseases [4]. *P. gingivalis* has not been found in either healthy subjects or patients with gingivitis [5]. Consequently, elevated levels of these putative pathogens may be useful indicators of both active periodontitis and increased risk of gingival attachment loss. However, knowledge of how their numbers relate to disease progression is still unclear and for longitudinal studies accurate assessment of their numbers in clinical samples is needed.

Several methods have been employed to detect putative periodontopathogens in clinical samples. These include

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cultural methods, microscopy, immunofluorescent assays, enzyme-linked immunosorbent assays, trypsin-like protease assays, DNA probes [6] and most recently the PCR [7]. Apart from the latter, all suffer either from lack of specificity or sensitivity, whereas PCR has been reported to detect as few as 50 cells of *P. gingivalis*, *Pr. intermedia* or *A. actinomycetemcomitans* [7]. Although PCR is sensitive, quantification of the target sequence by this method is difficult because of tube-to-tube variations in amplification efficiency and the kinetics of the enzyme reaction. Small variations in amplification efficiency can produce significant changes in product yield and so distort experimental data. Hence, reliable quantification should employ a reference standard and this forms the basis of the quantitative-competitive PCR (QC-PCR) method. QC-PCR involves co-amplification of a reference standard in the same reaction tube as the target DNA; therefore, both are subjected to identical conditions. By adding varying amounts of reference DNA, the amount of target in a sample can be determined by comparison with the amount of reference DNA that yields an equimolar quantity of PCR product. QC-PCR has previously been used to enumerate viral DNA, bacteria and parasites [8–10] and has more recently been employed to assess the accumulation of bacteria during an oral hygiene cessation programme with primers for the 16S rRNA gene [11]. However, the approach described here does not appear to have been applied to the study of dental plaque taken from subjects with periodontitis.

The aim of the present study was to develop a sensitive QC-PCR technique for the enumeration of *P. gingiva-*

lis, *Pr. intermedia* and *A. actinomycetemcomitans*, both laboratory strains and subgingival plaque samples, with primers specific for the *fimA* gene, the 16S rRNA gene and the *lktA* gene respectively.

Materials and methods

Cultivation of bacterial strains

P. gingivalis W50, *Pr. intermedia* ATCC 25611, *Pr. nigrescens* ATCC 25261 and 36 clinical isolates (12 each of *P. gingivalis*, *Pr. intermedia* and *Pr. nigrescens*) were cultured on Fastidious Anaerobe Agar (FAA; LabM, Bury) supplemented with horse blood 5% v/v, in an anaerobic jar in an atmosphere of N₂ 80%, H₂ 10% and CO₂ 10% at 37°C for 3–5 days. Each strain was then subcultured in Brain Heart Infusion Broth (BHIB; Oxoid) supplemented with haemin (Sigma) 5 mg/L and yeast extract (Sigma) 0.5% w/v. Ten strains of *A. actinomycetemcomitans* (ATCC 29522 and nine clinical isolates) and seven strains of *Haemophilus aphrophilus* (kindly supplied by Dr M. Riggio, Glasgow University) were cultured on Tryptic Soy Agar (Oxoid) in an atmosphere of CO₂ 10% in air at 37°C for 3–5 days. The resultant growth was then inoculated into BHIB and cultured at 37°C for 2 days. All broths were centrifuged at 12 000 *g* for 10 min and cell pellets were kept at –70°C until required. Other bacterial species and isolates used in the study were also prepared as appropriate (listed in Table 1).

Subgingival plaque samples were plated on to FAA for isolation of black-pigmented anaerobes or TSBV agar

Table 1. Identity and source of bacterial strains used in the study

Bacteria	Strain(s)	Source
<i>A. actinomycetemcomitans</i>	ATCC 29522 + 9 clinical isolates	Invitrogen
<i>Actinomyces naeslundii</i>	1 clinical isolate	
<i>Act. odontolyticus</i>	1 clinical isolate	
<i>Act. viscosus</i>	NCTC 10951	
<i>Bacteroides fragilis</i>	NCTC 9343	
<i>Capnocytophaga</i> sp.	OM 147	
<i>E. coli</i>	INFαF'	
<i>Eikenella corrodens</i>	1 clinical isolate	
<i>Fusobacterium nucleatum</i>	NCTC 11326	
<i>Fusobacterium</i> spp.	ATCC 25556, ATCC 25557, NCTC 10560, NCTC 10576, VPI 4877	
<i>Haemophilus aphrophilus</i>	NCTC 5886 + 6 clinical isolates	M. Riggio
<i>Leptotrichia buccalis</i>	NCTC 10249	
<i>P. endodontalis</i>	1 clinical isolate	R. Teanpaisan J. Slots
<i>P. gingivalis</i>	W 50 [†] + 12 clinical isolates	
<i>Pr. corporis</i>	ATCC 33547	D.E. Devine [†] , H.N. Shah [‡]
<i>Pr. denticola</i>	ATCC 35308	
<i>Pr. intermedia</i>	ATCC 26511 [†] , MH 7 [‡] + 12 clinical isolates	
<i>Pr. loescheii</i>	ATCC 15930	D.E. Devine [†] , H.N. Shah [‡]
<i>Pr. nigrescens</i>	ATCC 25261 [†] , MH 11 [‡] + 12 clinical isolates	
<i>Propionibacterium</i> spp.	1 clinical isolate	
<i>Pseudomonas aeruginosa</i>	NCTC 10662 + 1 clinical isolate	R. Russell
<i>Streptococcus mitis</i>	NCTC 10712	
<i>Str. mutans</i>	Ingbritt	
<i>Str. oralis</i>	NCTC 7864	
<i>Str. salivarius</i>	NCTC 8606	M. Kilian [§]
<i>Str. sanguis</i>	ATCC 10556 [§] , NCTC 7863	
<i>Str. vestibularis</i>	OV 71	

(bacitracin and vancomycin; Sigma) or the selective medium of Holm *et al.* [12] for *A. actinomycetemcomitans*. Plates were cultured under the appropriate conditions as described above for 3–7 days at 37°C. Strains of black-pigmenting anaerobes were classified as either *P. gingivalis*, by their ability to hydrolyse *N*-benzoyl-DL-arginine-2-naphthylamide (BANA; Sigma) and *p*-nitrophenyl- α -glucoside (Sigma) [13], or as *Pr. intermedia* by their fluorescence under long-wavelength UV light, ability to hydrolyse *p*-nitrophenyl- α -glucoside and whole-cell protein profiles on SDS-PAGE 12% [4]. Star-shaped colonies, presumptive of *A. actinomycetemcomitans*, were further identified by their ability to ferment raffinose and lactose and by production of catalase. No attempt was made to quantify the organisms cultured or to determine the detection limits of the culture method.

Dental plaque sampling

Subgingival plaque samples were collected from volunteer subjects with a sterile curette from sites with probing depths and attachment loss between 2–9 mm and placed in a sterile tube containing 700 μ l of reduced transport fluid (RTF) [14]. All volunteers gave written consent and ethical approval for the study was obtained from the South Yorkshire Ethics Committee. Each sample was washed once and resuspended in 250 μ l of sterile distilled water immediately after sampling, and then disrupted by ultrasonication at 25 W for 30 s, including two 5-s intervals. A 100- μ l volume of each sample was processed for DNA preparation as described below.

PCR

A 1-ml (10^6 cells/ μ l) volume of suspension of each reference strain or isolate (Table 1) was centrifuged, washed once and resuspended in 100 μ l of sterile distilled water. The plaque samples and the reference bacterial suspensions were heated in a microwave oven for 10 min then placed on ice. Cell debris was removed by centrifugation and the supernate was used for PCR, which was performed essentially as described by Saiki [15]. Briefly, 1 μ l of the supernate was added to 49 μ l of PCR master mixture – 5 μ l of 10 $\times$ buffer, pH 8.3, *Taq* DNA polymerase (Advanced Biotech, Surrey) 1.0 unit, 2.5 mM MgCl₂, 0.2 mM of each dNTP (Promega, Southampton), 0.1 μ M of each primer pair (Perkin Elmer, Warrington) – and overlaid with light mineral oil. The reaction sequence consisted of 30 amplification cycles, each comprising dissociation of DNA (95°C), annealing of primer (55°C) and primer extension (72°C) for 1 min each. Initial dissociation of DNA was for 5 min and final extension was for 10 min at 72°C. PCR products were stored at –20°C or analysed immediately by electrophoresis on agarose 2.5% gels (Agar-ease MP, Scotlab, Coatbridge) in 1 $\times$ TAE buffer (40 mM Tris base, 40 mM acetic acid, 1 M EDTA) for 45–60 min at *c.* 5 V/cm. Gels were then stained with

ethidium bromide 0.5 mg/L and photographed. Reference strains and clinical isolates, representing 14 genera and 32 species (Table 1), were tested for the specificity and sensitivity of the PCR primers. To control for possible bacterial DNA contamination in the sterile distilled water used, samples of the water were either filtered through a 0.45- μ m filter or not filtered and each was added to a separate PCR reaction mixture without any DNA solution before amplification. To study possible interference of plaque components in the PCR, *P. gingivalis* W50, *Pr. intermedia* ATCC 25611 or *A. actinomycetemcomitans* ATCC 29522 (10^5 cells) were resuspended in 50 μ l of sterile distilled water and each was added to suspensions of 50 μ l of dental plaque samples that were negative for these three organisms in previous tests. The mixtures were then run in PCR assays and the amount of product was compared to the amount obtained from PCR conducted with the same number of cells of each strain alone.

Construction of competitive templates for QC-PCR

Competitive templates for each of the three target species were constructed by cloning appropriate DNA fragments into plasmids. Details of the primers employed and strategy for template construction are given in Table 2 and Fig. 1, respectively. The PCR product from the competitive templates must be a different size from the product amplified from the target template. In the case of *P. gingivalis*, no unique sequence with appropriate G+C mol % content suitable for a competitive template could be found within the *P. gingivalis* *fimA* gene, either upstream or downstream of the *P. gingivalis* forward and reverse primer binding sites, so a competitive template was constructed by ligating a 5' extension specific for these primer binding sites on to a sequence from the *A. actinomycetemcomitans* *lktA* gene, which did have appropriate G+C content. The resultant 232-bp PCR product, generated by the PgCf and the PgCr primers, was cloned into the vector pCRTMII (Invitrogen, Groningen, The Netherlands) and called plasmid-*P. gingivalis* (pPg). A combined competitive plasmid termed pAi was constructed for both *A. actinomycetemcomitans* and *Pr. intermedia*. This was created by ligating the PCR products produced with primers AaCf and AaCr (311 bp) with those produced with primers PiCf and PiCr (247 bp) and then cloning the resultant ligation fragment in the vector pCRII. Both the pAi and pPg competitive plasmids were constructed with the Original TA Cloning Kit (Invitrogen) and after plasmid purification (Promega), each plasmid was stored at –20°C until further use. To confirm that the cloned sequences were inserted into the plasmid, each plasmid was amplified by each primer pair under the basic PCR conditions described above. PCR products were then purified with the PCR Purification Kit (Promega) and processed for automated sequencing. Sequence alignment analysis of the inserted sequences and the

Table 2. PCR primers used in the study

Primers for competitive template construction					
Target gene	Design(s) for	5'-extension for 'working primer' [*]	2° primer† (5'-3')	Name	Position
<i>ltxA</i> gene (Aa)	<i>P. gingivalis</i> , competitor	CCCGGGATCTGAACGAAGT GCGAC	GAATATCGTAACTATAAA	PgCf	2596–2613
	<i>P. gingivalis</i> , competitor	GGCCGTTCTGCCTCGTTGTCTTTTA	ATACAAACGATCATCGCCAT	PgCr	2790–2771
	<i>A. actinomycetemcomitans</i> , competitor	GTACGGGAAGGTTACCGATCTACTTA	AATTATCGGCAGCACACTA	AaCf	2677–2694
	<i>A. actinomycetemcomitans</i> , competitor	CCCGGC	TTGCCACTTCCACCTCTT	AaCr	2966–2949
16S rRNA gene (Pi)	<i>Pr. intermedia</i> , competitor	GGCCGTCCACATATGGCATCTGCGCG	TTGTTGGGGAGTAAAGCG	PiCf	413–430
	<i>Pr. intermedia</i> , competitor	GTAC	ATACGTTGCGTGCACTCA	PiCr	638–621
Primers for QC-PCR					
Target gene	Function(s)	5'-extension	Working primer [*] (5'-3')	Name	Position
<i>fimA</i> gene (Pg)	<i>P. gingivalis</i> , detection	–	ATCTGAACGAAGT GCGAC	Pgf	2–19
	<i>P. gingivalis</i> , detection	–	GTTCTGCCTCGTTGTCTT	Pgr	172–155
<i>ltxA</i> gene (Aa)	<i>A. actinomycetemcomitans</i> , detection	–	GGGAAGGTTACCGATCTA	Aaf	2575–2592
	<i>A. actinomycetemcomitans</i> , detection	–	TTGCCACTTCCACCTCTT	Aar	2966–2949
16S rRNA gene	<i>Pr. intermedia</i> , detection	–	GTCCACATATGGCATCTG	Pif	153–170
	<i>Pr. intermedia</i> , detection	–	ATACGTTGCGTGCACTCA	Pir	638–621
	Total bacteria, detection	–	AGAGTTTGATCMTGGCTCAG	27f	27–46
	Total bacteria, detection	–	CTGCTGCSYCCCGTAG	342r	342–327

^{*}Binding site of the designed primer for detection of each specified bacterium (in bold type).

[†]Binding site of a secondary primer used to create a 5' extension for insertion of the designed primer and DNA core template into a plasmid.

Fig. 1. Target and competitive template designs for *P. gingivalis*, *Pr. intermedia* and *A. actinomycetemcomitans*. A heterologous DNA fragment (e.g., region of the *lktA* gene) was amplified with secondary primers composed of a 3' end that anneals to the target sequence (□) and a 5' end that consists of working primers (■). The PCR product obtained, tagged by the specific working primers (e.g., Pgf or Pgr), was then cloned into the pCRII vector.

published gene sequences was used to compare the similarity of DNA sequences (Align Plus, Sequences Alignment Program version 2.0, Scientific & Educational Software, UK).

For use in the QC-PCR, competitive plasmid templates were transformed into competent *Escherichia coli* cells and screened by blue-white colony transformation. Control samples of known *E. coli* cell number were then added to PCR reactions as appropriate.

QC-PCR

The competitive PCR was performed as described in the basic PCR procedure above (summarised in Fig. 2), but with supernate from 10^3 microwave-heated cells of *P. gingivalis*, *Pr. intermedia* or *A. actinomycetemcomitans* (four replicates of 1 μ l) mixed with 1 μ l of each of 10-fold serial dilutions of 10^6 appropriate transformed *E. coli* cells in a 50- μ l reaction volume. PCR products were prepared for automated sequencing as described above for confirmation of sequences.

To determine copy numbers of each target/competitive template pair [16,17], each bacterial suspension was diluted to the same concentration of 10^6 cells and replicates (1 μ l) of each of the target template and the competitive template suspension were added to PCR mixtures and amplified for 18, 20, 21, 22, 23, 24, 25 and 27 cycles. PCR products were electrophoresed as described above.

Enumeration of *P. gingivalis*, *Pr. intermedia* and *A. actinomycetemcomitans* in subgingival plaque samples

A mixture of 1 μ l of each plaque supernate and 1 μ l of 10-fold serial dilutions of 10^6 transformed *E. coli* cells was mixed with the PCR reaction master mix to make a final volume of 50 μ l. Samples were then subjected to PCR and gel electrophoresis and analysed with IS-1000 Digital Imaging System software (Alpha Innotech Corporation, San Leandro, CA, USA). The fluorescence values were adjusted by subtracting the background gel fluorescence from each target PCR product and each competitor. For determining the ratio of the

copy numbers of the target template and the competitive template, the average (triplicate determinations) fluorescence values per number of base-pairs in each fragment were calculated, because ethidium bromide binds to DNA by intercalation between the base planes of the DNA helix. The logarithm of this fluorescence value for each target/competitor pair was then plotted against the PCR cycle number.

For enumeration of *P. gingivalis*, *Pr. intermedia* and *A. actinomycetemcomitans*, the fluorescence was then corrected for size and copy number difference. The ratio of the corrected fluorescence of each target to its competitor was plotted against the known competitor input and the equivalence point was calculated as the value where the target was equal to that of the competitor.

Enumeration of total bacteria in samples

The total number of bacteria in plaque samples was determined by quantitative PCR (Q-PCR) with primers to the 16S rRNA gene. The primers chosen were 27f, 342r, 357f, 1100r and 1492r, from published sequences with the *E. coli* numbering [18]. They were matched into five pairs: 27f and 342r; 27f and 1100r; 27f and 1492r; 357f and 1100r; and 357f and 1492r. To test these primer pairs, bacteria listed in Table 1 were examined by the basic PCR as described above. The primer pair 27f and 342r proved to be the most useful and so was used for estimating the total number of bacteria in all plaque samples. To determine the effect of any variation in copy number of the 16S rRNA gene of different organisms, serial dilutions (up to 10^6 cells) of each species listed in Table 1 were subjected to PCR in separate tubes under the same amplification conditions.

Uniform amplification of mixtures of DNA with varying G+C content can be problematic and to try to resolve this, several variations of the procedure were tested. These included: boiling the template before amplification (1–10 min) [19]; denaturing with 0.4 M NaOH and 0.4 mM EDTA for 10 min at room temperature followed by DNA purification with a commercial DNA clean-up kit (Promega) [20]; adding

Fig. 2. Protocol for performing QC-PCR.

glycerol (5–10%), DMSO (0.2–20%) or betaine (0.5–5 M, Sigma), both separately and in combinations [21–24].

For enumeration of total bacteria present in plaque samples, the PCR reaction master mix was added separately to 1 μ l of each boiled plaque sample and to 1 μ l of 10-fold serial dilutions of 10^6 *E. coli* cells (strain INF α F') in separate tubes. The basic PCR conditions were modified as follows; 3 mM MgCl₂ and 22 repeated cycles of 95°C (2 min), 50°C (2.5 min) and 72°C (2 min). The logarithm of the fluorescence intensity of PCR products was plotted against the log₁₀ of the cell input and used to interpolate the number of bacteria in each plaque sample. By reference to a standard calibration curve produced from serial 10-fold dilutions of known numbers of *E. coli*, the number of bacteria in each plaque sample was computed.

Enumeration of total bacteria in subgingival plaque samples by phase-contrast microscopy

The QC-PCR method was compared with microscopy for determination of total numbers of bacteria in samples. Each sample was counted by phase-contrast microscopy with a counting slide (Thoma, Hawksley) at a magnification of $\times 400$ within 1 h of sampling, by a single investigator and repeated three times.

Statistical analysis

Linear regression analysis was used to calculate the bacterial numbers in the plaque samples, at the equivalence point of the target cells and the reference cell inputs. The Kruskal-Wallis H test was employed to determine the effect of copy number of the bacterial 16S rRNA gene. The Wilcoxon signed ranks test was used to compare the number of bacteria present in subgingival plaque samples obtained by Q-PCR and phase-contrast microscopy.

Results

PCR

After 30 amplification cycles, the primers for *P. gingivalis* (Pgf and Pgr), *Pr. intermedia* (Pif and Pir) and *A. actinomycetemcomitans* (Aaf and Aar) generated a single amplicon of expected size, i.e. 172 bp, 487 bp and 392 bp, respectively (Fig. 3). The sequence of these PCR products was 94–97% similar to the published sequences. The lowest detection levels for the three species were 100 *P. gingivalis* cells, 100 *Pr. intermedia* cells and 10 *A. actinomycetemcomitans* cells (data not shown). No cross-reactivity was found with any of the designed primers among the three target species or among any of the 14 genera of oral bacteria tested (listed in Table 1). Constituents in oral plaque samples did not interfere with the PCR detection, as the expected PCR band intensity was

obtained when cells of each of the three species were mixed with plaque samples that were negative for the target species. With the 27f and 342r 16S rRNA primer pair, only a slightly lower fluorescence intensity of the PCR products was found with filtered water than with non-filtered water, indicating the presence of some water bacteria before filtering. However, this contamination had little effect on the overall level of PCR products.

Competitive templates

The pAi plasmid carried an inserted ligation product of a 247-bp sequence from the 16S rRNA gene of *Pr. intermedia* along with a 311-bp sequence amplified from the *lktA* gene of *A. actinomycetemcomitans*. Therefore, the combined sequence could be recognised by both the Aaf and the Pif primers each at one end and included the Aar and the Pir primer binding sites in between. Each separate sequence had a homologous core sequence that was smaller but with similar G+C mol % composition to each target template. The initial aim was to construct a single plasmid that contained the competitive template inserts of all the three target species. However, despite several attempts, a single ligation product of the three target sequences could not be obtained. Consequently, a separate construct was made for the *P. gingivalis* competitive template. This template had to be constructed by ligating the *fimA* primer binding sites to a non-homologous core because the G+C mol % content of DNA sequences upstream and downstream of the *P. gingivalis* primer binding sites in the *fimA* gene was not appropriate. The Pgf and Pgr primer binding sites, one at each 5'-extremity, were ligated to a 232-bp sequence from the *A. actinomycetemcomitans lktA* gene. This sequence did have an appropriate G+C mol % content and did not contain the primer binding sites for detecting *A. actinomycetemcomitans*. Therefore, the resultant plasmid, pPg, contained a non-homologous insert of a different size but with similar G+C mol % content to the target template.

In the preliminary work to check the validity of the primers, no difference in the efficiency of the primers was found when amplifying either a non-homologous or a homologous core template that included the primer binding sites [25]. Also, differences in the length of the DNA sequences between the target and competitive templates had no or little effect on their relative amplification efficiency.

The copy numbers of the target and competitor sequences for *Pr. intermedia* and *A. actinomycetemcomitans* were not the same. The ratios of *P. gingivalis* and its competitive template, *Pr. intermedia* and its competitive template, and *A. actinomycetemcomitans* and its competitive template were found to be 1:1, 2:3 and 1:3 respectively.

Fig. 3. Agarose gel electrophoresis of QC-PCR products. (a) *P. gingivalis* and competitive template (pPg). Lane 1, 100-bp mol. wt markers; 2, 6600 *P. gingivalis* cells; 3–6, 7, 660, 6.6×10^4 and 6.6×10^6 *E. coli* cells containing pPg co-amplified with 6600 *P. gingivalis* cells respectively; 7, negative control (no DNA); 8, 100-bp mol. wt markers. (b) *A. actinomycetemcomitans* and competitive template (pAi). Lane 1, 100-bp mol. wt markers; 2, negative control (no DNA); 3–6, 7, 660, 6.6×10^4 and 6.6×10^6 *E. coli* cells containing pAi co-amplified with 6600 *A. actinomycetemcomitans* cells respectively. *Pr. intermedia* and competitive template (pAi). Lane 7, negative control (no DNA); 8–11, 7, 660, 6.6×10^4 and 6.6×10^6 *E. coli* cells containing pAi co-amplified with 6600 *Pr. intermedia* cells respectively.

Quantitative PCR technique

The competitive templates employed in this study were total DNA suspensions without plasmid extraction prepared from appropriate recombinant *E. coli* by microwave heating. This was because preliminary work

indicated that trace amounts of whole genomic DNA contaminated plasmid preparations produced by means of a commercial kit (Promega) and because this extraction procedure more closely mimicked the extraction of the target DNA template from plaque samples.

A 1- μ l volume of DNA suspension from 10^3 *P. gingivalis* cells and 1 μ l of each of 10-fold serial dilutions of pPg plasmid from up to 10^6 transformed cells were co-amplified. Thirty cycles of PCR amplification were performed, allowing the reaction to reach a plateau. As expected, a 172-bp and a 232-bp DNA product were obtained (Fig. 3) showing that amplification of both templates had occurred. Similar expected results were obtained when co-amplifying *Pr. intermedia* or *A. actinomycetemcomitans* and the pAi competitive template (Fig. 3). A calibration curve for each co-amplification was constructed from data obtained by densitometric analysis of the electrophoretic patterns. A $\log_{10}$ plot of the target/competitor PCR product ratio as a function of numbers of competitor *E. coli* cells yielded straight lines, as shown in Fig. 4 and Table 3 shows data comparing actual and computed cell numbers of bacteria. To validate the QC-PCR, a fixed number of competitive cells or reference cells (10^3) was added and the equivalence between them was established. This always turned out to be approximately the expected 10^3 cells no matter which of the reference

or competitive cells were fixed, indicating that the QC-PCR method for estimation of the absolute amount of unknown DNA was reliable.

When enumerating the total bacteria present in a sample, each of the 16S rRNA primer pairs generated the expected PCR products. However, the 27f and 342r primer pair proved to be the most sensitive, yielding a single and distinct DNA band of 316 bp from as few as 100 bacteria (Fig. 5). With these primers and the same number of cells of each of the bacterial strains listed in Table 1, little or no difference in PCR band intensities (1.1 SD 0.3-fold of the band intensity produced by *E. coli*, data not shown) was found. One exception to this was for the *Actinomyces* spp. that yielded lower than expected amounts of PCR product. This observation was probably due to the high G+C content of the genus *Actinomyces* [26] and so several modifications to the DNA processing procedure were investigated to try to improve the amplification of DNA from this genus. Of these modifications, boiling the templates for 10 min and NaOH denaturation of the templates before

Fig. 4. Curve fit by linear regression analysis of (a) *P. gingivalis* ($y = 1.1 - 0.3x$, $r = 0.981$), (b) *Pr. intermedia* ($y = 1.4 - 0.4x$, $r = 0.998$) and (c) *A. actinomycetemcomitans* ($y = 0.76 - 0.2x$, $r = 0.944$) by QC-PCR and (d) the total number of bacteria with *E. coli* as the reference species ($y = 0.28 - 0.4x$, $r = 0.996$) by closed-tube Q-PCR; ---*---, observed; -□-, estimated (a,b,c) or linear (d).

Table 3. Comparison of actual and estimated cell numbers from triplicate runs

Bacteria	Experimental gradient	r	Input cells	Estimated cells; mean (SD)
<i>P. gingivalis</i>	0.3	0.99	6600	5420 (860)
<i>Pr. intermedia</i>	0.4	0.99	6600	5360 (180)
<i>A. actinomycetemcomitans</i>	0.2	0.99	6600	8490 (1010)
Total bacteria sample 1	0.4	0.99	7	16 (10)
Total bacteria sample 2	0.4	0.99	6600	4731 (520)
Total bacteria sample 3	0.4	0.99	660 000	407 970 (138 200)

Estimated cells calculated from a curve fit produced by linear regression analysis to assess the accuracy of the method (see Fig. 4).

Fig. 5. Quantitative PCR for total bacterial counts with 16S rRNA 27f and 342r primers. Lanes 1–7, 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , 10^2 and 10^1 *E. coli* cells, respectively; 8, negative control (no DNA). The position of the predicted DNA fragment is shown.

PCR aided the amplification of *Actinomyces* spp., but without any or with only slightly increased PCR yield from other genera.

Enumeration of P. gingivalis, Pr. intermedia, A. actinomycetemcomitans and total bacteria in plaque samples

The QC-PCR method described above was employed to enumerate the three target species in subgingival plaque samples. Both the expected target and competitive PCR products were obtained and no other bands were found. The PCR products that were sequenced showed 94–97% similarity to the published sequences (GenBank), with the single exception of the product obtained from the pAi competitive template sequenced with the Aaf primer, which was 86% similar.

To validate PCR-positive results from plaque samples,

12 samples were randomly selected from adult periodontitis patients who participated in the study and part of each sample was processed for PCR and part was cultured. Experiments were repeated in triplicate. These 12 subgingival plaque samples were collected from sites in separate patients with probing depths of 2–9 mm. The total number of bacteria determined by PCR ranged from 1.2×10^6 to 5.5×10^8 cells/ μ l. The numbers of *P. gingivalis*, *Pr. intermedia* and *A. actinomycetemcomitans* were 0 – 8.3×10^4 , 0 – 6.8×10^5 and 0 – 1.1×10^6 cells/ μ l, respectively (Table 4). Of the 12 plaque samples studied, *Pr. intermedia* was found by PCR in six samples but in only two by culture; *P. gingivalis* was detected by PCR in six samples but only one of these was culture-positive and *A. actinomycetemcomitans* was found by PCR in eight samples but none was culture-positive. To improve the possibility of detection of *A. actinomycetemcomitans* in clinical samples by culture, pooled plaque samples from several sites within each of five adult periodontitis

Table 4. Number of *P. gingivalis* (Pg), *Pr. intermedia* (Pi) and *A. actinomycetemcomitans* (Aa) cells (mean of three determinations) and proportion of total count in subgingival plaque samples determined by QC-PCR

Sample no.	Pocket depth (mm)	Mean total bacterial count (cells/ μ l)	Mean Pg count/ μ l (% of total count)	Mean Pi count/ μ l (% of total count)	Mean Aa count/ μ l (% of total count)
1	9	156798 500 SD 21 451 500	50900 SD 3060 (0.03 SD 0.01)	378 000 SD 30 400 (0.27 SD 0.22)*	7330 SD 700 (0.01 SD 0.00)
2	8	511934 100 SD 39 547 900	0 SD 0 (0)	26720 SD (2010 (0.01 SD 0.00)	2910 SD 30 (0)
3	7	2381300 SD 85800	50 SD 10 (0)	1480 SD 110 (0.06 SD 0.00)	0 SD 0 (0)
4	7	1321400 SD 115400	0 SD 0 (0)	0 SD 0 (0)	0 SD 0 (0)
5	6	2949600 SD 154950	79 750 SD 2900 (2.71 SD 0.24)*	0 SD 0 (0)	8800 SD 310 (0.30 SD 0.01)
6	4	10275000 SD 440 360	0 SD 0 (0)	6790 SD 310 (0.07 SD 0.01)*	11 020 SD 890 (0.11 SD 0.01)
7	4	7549700 SD 272 300	0 SD 0 (0)	0 SD 0 (0)	0 SD 0 (0)
8	4	4637000 SD 107 600	10 SD 10 (0)	0 SD 0 (0)	1 045 100 SD 75 000 (22.51 SD 1.10)
9	3	3 174 600 SD 260 200	470 SD 40 (0.02 SD 0.00)	550 SD 30 (0.02 SD 0.00)	21 220 SD 1280 (0.73 SD 0.08)
10	3	1742650 SD 251 400	0 SD 0 (0)	270 SD 50 (0.01 SD 0.00)	3080 SD 240 (0.18 SD 0.04)
11	3	10 314 580 SD 961 920	0 SD 0 (0)	0 SD 0 (0)	3680 SD 240 (0.04 SD 0.01)
12	2	1 500 780 SD 162 230	270 SD 40 (0.02 SD 0.01)	0 SD 0 (0)	0 SD 0 (0)

*Culture positive.

patients were collected separately. One of these was culture-positive for *A. actinomycetemcomitans*, whereas all five were positive by PCR (data not shown). No attempt was made to measure the sensitivity of the culture method.

The Q-PCR method for enumerating the total number of bacteria was compared with phase-contrast microscopy with 108 subgingival plaque samples. The mean number of bacteria estimated by the PCR was approximately five times higher than that determined by microscopy, with the discrepancy ranging from 4×10^3 to 2×10^5 cells. However, the data generated by the two techniques were in general agreement, in so far as those samples that gave high numbers by PCR also gave high numbers by microscopy.

Discussion

This study employed a PCR technique to quantify *P. gingivalis*, *Pr. intermedia* and *A. actinomycetemcomitans* in subgingival plaque samples. This method proved to be rapid, reproducible and extremely sensitive and the primers employed were highly specific and capable of differentiating the target organisms from a number of closely related cultivable species (such as *A. actinomycetemcomitans* from *H. aphrophilus* and *Pr. intermedia* from *Pr. nigrescens*). The primers chosen for detection of the three species were based on virulence genes and the 16S rRNA gene. The *fimA* gene is considered to be present in all strains of *P. gingivalis*, to be entirely specific and previous work had involved use of primers for this gene. Similarly, the *lktA* gene of *A. actinomycetemcomitans* is considered to be present in all strains of *A. actinomycetemcomitans* and is specific to this species. For these reasons the *fimA* and *lktA* genes seemed suitable candidates upon which to base a quantitative PCR method. However, there are few data concerning genes for *Pr. intermedia* and so the 16S rRNA target appeared to be the best option for detecting this species.

The *fimA* gene of *P. gingivalis* has been studied by several workers [27–30]. Some *P. gingivalis* strains, including strains W50, W83 and AJW5, are known to be poorly fimbriate, whereas others strains, such as 381, ATCC 33277 and HG 565, are highly fimbriate and the latter have significant adhesive abilities to host tissues and salivary components [27, 28]. However, regardless of the expression of fimbriae, all *P. gingivalis* strains have been reported to possess the structural gene, *fimA*. Takahashi *et al.* [29] found that all of 29 *P. gingivalis* strains tested possessed a single copy of the *fimA* gene on their chromosomal DNA and no homologous sequences have been found within other known species, although intra-strain variation in the structure of the *fimA* gene is known [30]. The type strain of *P. gingivalis* was not included in the present study but strain W50, a frequently used reference

strain, was employed as well as 12 clinical isolates. The *fimA* primers described here were able to amplify the target region of all these 13 *P. gingivalis* strains. Alignment of the published sequences of the *fimA* gene (GenBank) of *P. gingivalis* strains W50, 381, ATCC 33277, ATCC 4 9417, HW24D1, OMZ314 and OMZ409 was also performed and data showed that the DNA region chosen here for the *fimA* primers was highly conserved amongst all the strains. Furthermore, the region was not complementary to the related species, *P. asaccharolyticus* (ATCC 25260).

A. actinomycetemcomitans produces various potential virulence factors including a proteinaceous leukotoxin [31, 32] belonging to the RTX (repeats-in-toxin) family of bacterial cytolysins [33]. It is encoded from an operon containing four genes, named *lktA*, *lktB*, *lktC* and *lktD*. The *A. actinomycetemcomitans* primers used here were designed to amplify part of the *lktA* gene and were modified from previously described primers [34]. Again, this gene is present in all strains irrespective of the expression of toxin [35] and other species with cytotoxic genes of similar structure do not contain *lktA* homologous sequences [36]. The results of the present study support these findings, with all 10 strains of *A. actinomycetemcomitans* yielding the predicted PCR product, but no product being obtained from seven *H. aphrophilus* strains. *Pr. intermedia* primers were chosen from the 16S rRNA gene, which has been studied by several workers [7, 37, 38]. The *Pr. intermedia* primers amplified the expected sequence from all 14 strains of *Pr. intermedia* but yielded no amplicons with 14 strains of the closely related species, *Pr. nigrescens*, or with any other species under the chosen conditions. Unfortunately, strains of the new species *Pr. pallens* [39] were not available, but the primer sequences did not align with the published 16S rRNA sequences of three strains (9423, 8792 and 10371) of this species. However, there was alignment with five bases at the 3' end of the reverse primer and the same region of *Pr. pallens* 16S rRNA sequences. This could possibly be problematic under less stringent PCR conditions than those used here. Indeed, in preliminary work in which less stringent conditions were used, the primers employed were able to amplify the *Pr. nigrescens* 16S rRNA gene, which is very similar to *Pr. pallens* (GenBank), but increasing the stringency abolished this cross-reactivity. Thus, although the data indicate that cross-reactivity was not a problem here, there may be potential difficulties basing QC-PCR on 16S rRNA genes when trying to quantify a target species among a complex mixture of bacteria.

For QC-PCR, competitive templates are required which yield different sized amplicons from that amplified from the target bacterial sequence, because they need to be separated by electrophoresis. The results showed that the *P. gingivalis*, *Pr. intermedia* and *A. actinomycetemcomitans* primers used could recognise both the target gene and the competitive templates that were

constructed. However, the amount of PCR products derived from one of the competitive plasmids, pAi, was more than that from the target gene; one and a half and three times as much for *Pr. intermedia* and *A. actinomycetemcomitans*, respectively. This difference was not due to variation in the amplification efficiency of the target and competitor sequences due to their size, because the data were adjusted for any effects of size difference. However, there are two other possible explanations. It is possible that more than one competitive plasmid was present in the transfected *E. coli* cells. Alternatively, more than one copy of the gene could have become ligated into the competitive plasmid template, so that the number of copies of the gene per *E. coli* cell would be higher than the copy number of the original gene in the reference bacterial cells. It is not known which of these possibilities is the explanation, but the data were adjusted to take account of these copy number ratios and showed that in all replicates (three runs) the computed numbers of the transfected competitive *E. coli* cells were close to the actual *P. gingivalis*, *Pr. intermedia* or *A. actinomycetemcomitans* cell input when the competitors were used to estimate the target cell number or when the reverse was performed, using the targets to enumerate the competitors. This indicates that the assay was reproducible.

To determine the proportions of *P. gingivalis*, *Pr. intermedia* and *A. actinomycetemcomitans* in plaque samples rather than the absolute number, the ability to determine the total number of bacteria present was needed. For this, primers from the 16S rRNA gene were used, which have been reported to be highly conserved in eubacterial species [18] and of which there are four-to-six copies per cell (e.g., six copies for *E. coli*) [40]. These were matched into five primer pairs, each of which gave the expected PCR product; however, only the 27f and 342r primer yielded a single and distinct band. The study examined the sensitivity of this primer pair with bacteria representing 14 genera (Table 1) and obtained both the expected amplicon product and the same amount of product per cell from all species tested, with the exception of *Actinomyces* species. The lower yield of PCR product from this genus was probably due to the high G+C content of the chromosome, but was overcome by modifying the PCR conditions. This indicates that the difference in copy number of this region has no effect on enumerating the total number of bacteria.

As part of the validation of the quantitative PCR assay, the study compared the total number of bacteria obtained by phase-contrast microscopy with that obtained by QC-PCR and the recovery of the three target organisms by culture. Overall, there was concordance between the methods, but the PCR method was found to be 3–10-fold more sensitive than microscopy and more sensitive than culture, although no attempt was made to determine the

sensitivity or to quantify the organisms by culture. These findings are in agreement with those of other workers [7, 41–43].

The QC-PCR was successful in quantifying the three target species in subgingival plaque samples and the data showed variations in their numbers and frequencies. However, because the data derived from only 12 treated sites, it is not possible to draw conclusions about any relationship between these organisms and clinical parameters of the disease, but the study is being widened. Despite the usefulness of QC-PCR, some workers consider 'Real Time' assays such as those performed on the 'TaqMan' equipment (Perkin Elmer-Cetus) to be more effective, but QC-PCR has been shown to give similar accuracy and reliability to the TaqMan assay [44]. Also, such commercial systems have the disadvantages of requiring expensive instrumentation, the construction of complex probes and the requirement to adjust the PCR conditions to optimise the hybridisation efficiency of the probe.

In summary, the present study showed the applicability of quantitative-PCR for accurately determining the numbers of *P. gingivalis*, *Pr. intermedia* and *A. actinomycetemcomitans* both with laboratory cultures and in clinical plaque samples. Such quantification is needed for longitudinal studies on the change in numbers of these organisms in relation to disease onset, progression and response to treatment. The method was significantly more sensitive than culture and published data suggest that it is much more sensitive than other quantitative assays that have been applied to the detection of periodontopathogens. Determination of the numbers of these three species in subgingival sites in relation to clinical parameters of periodontitis will be reported separately.

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