Direct quantitative differentiation between *Prevotella intermedia* and *Prevotella nigrescens* in clinical specimens

Rudolf Gmür and Thomas Thurnheer

Author for correspondence: Rudolf Gmür. Tel: +41 1634 3306. Fax: +41 1634 4310.
e-mail: gmuer@zzmk.unizh.ch

Institute for Oral
Microbiology and General
Immunology, Centre for
Dental, Oral Medicine and
Maxillofacial Surgery,
University of Zürich,
Plattenstrasse 11, CH-8028
Zürich, Switzerland

This paper describes a quantitative fluorescent *in situ* hybridization (FISH) assay for the differential identification of *Prevotella intermedia* and *Prevotella nigrescens* in clinical samples, and compares its performance with less discriminatory culture and quantitative immunofluorescence (IF) assays. Fluorescence-labelled oligonucleotide probes directed to specific 16S rRNA sequences of *P. intermedia*, *P. nigrescens*, *Prevotella pallens* and *Prevotella denticola* were hybridized under stringent conditions with cultured reference strains or plaque samples from deep periodontal pockets. Probe specificity was defined with strains from multiple oral *Prevotella* species. The lower detection level of the assays was approximately 3×10^3 target cells per ml of plaque-sample suspension. *P. intermedia*, *P. nigrescens*, *P. pallens* and *P. denticola* were detected in plaques with prevalences of 69, 67, 0 and 28%, respectively. On average, 3.9×10^6 *P. intermedia*, 3.1×10^6 *P. nigrescens* and 5.6×10^5 *P. denticola* cells were counted per positive sample. All three species were found almost exclusively in dense mixed aggregates. Quantitative FISH data agreed satisfactorily with corresponding IF data ($r=0.711$). Both FISH and IF enumerations of the sum of *P. intermedia* and *P. nigrescens* markedly exceeded the c.f.u. counts of black-pigmented colonies in *Porphyromonas gingivalis*-free cultured subgingival plaques. The results demonstrate the validity of this new assay. Unlike established IF, culture, PCR or checkerboard DNA hybridization assays, this FISH assay differentiates quantitatively between *P. intermedia* and *P. nigrescens*, provides visual accuracy control, and offers insights into the spatial distribution of the target cells within a clinical sample.

Keywords: fluorescent *in situ* hybridization, 16S rRNA, subgingival plaque, periodontitis

INTRODUCTION

The human oral cavity harbours a variety of moderately saccharolytic anaerobic species of the bacterial genus *Prevotella*, some of which may have clinical significance. The two most prevalent species of the group are *Prevotella intermedia* and *Prevotella nigrescens*. Formerly considered a single species, they were separated 10 years ago (Shah & Gharbia, 1992; Frandsen *et al.*, 1995) after multiple DNA homology studies (van Steenberg *et al.*, 1982; Johnson & Holdeman, 1983; Moncla *et al.*, 1988; Fukushima *et al.*, 1992), serological experiments

(Gmür & Wyss, 1985; Nakazawa *et al.*, 1988), isoenzyme screenings (Shah & Gharbia, 1992; Frandsen *et al.*, 1995) and whole-protein analyses (Shah & Gharbia, 1992) had provided evidence for at least two distinct subgroups. Several investigations reported significant differences in the prevalence and/or abundance of the two species in samples collected from various oral sites (Moore *et al.*, 1987; Dahlén *et al.*, 1990; Fukushima *et al.*, 1992; Gharbia *et al.*, 1994; Gmür & Guggenheim, 1994; Baumgartner *et al.*, 1999). For example, when investigating the predominant cultivable microbiota in samples from adults, Moore *et al.* (1987) detected five times as many *P. intermedia* as *P. nigrescens* c.f.u. in periodontal pockets, but found seven times more *P. nigrescens* than *P. intermedia* at healthy sites. Or, in a

Abbreviations: FISH, fluorescent *in situ* hybridization; IF, indirect immunofluorescence.

study leading to similar conclusions, Dahlén *et al.* (1990) identified 2/3 of their *P. intermedia*-like isolates from 'destructive periodontal disease' as *P. intermedia*, whereas 75% of the isolates from healthy control subjects were *P. nigrescens*. Such findings lead to the hypothesis that *P. intermedia* could be a periodontal pathogen (reflected by its inclusion as an indicator organism in several microbiological diagnostic kits for periodontal diseases), whereas *P. nigrescens* is considered a marker of relative periodontal health.

Phenotypically, the two species are very similar. Biochemical or serological differentiation is difficult, laborious and sometimes unreliable (Gmür & Wyss, 1985; Shah & Gharbia, 1992; Gharbia *et al.*, 1994; Frandsen *et al.*, 1995; Dahlén *et al.*, 1996; Debelian *et al.*, 1996; Mättö *et al.*, 1996b; Paquet & Mouton, 1997; Baumgartner *et al.*, 1999; Lie *et al.*, 2001). Therefore, investigations now employ semi-quantitative checkerboard DNA probe assays (Socransky *et al.*, 1994) or PCR-based assays, of which multiple variations have been described, to differentiate between *P. intermedia* and *P. nigrescens*. So far, PCR-based assays have yielded only qualitative data, that is they were used either to type clonal black-pigmented colonies derived from primary cultures (Mättö *et al.*, 1996a, b; Conrads *et al.*, 1997; Guillot & Mouton, 1997; Baumgartner *et al.*, 1999; Haraldsson & Holbrook, 1999; Premaraj *et al.*, 1999; Robertson *et al.*, 1999) or to directly determine the presence or absence of the two species in clinical samples (Ashimoto *et al.*, 1996; Riggio *et al.*, 1998; Stubbs *et al.*, 1999; Mullally *et al.*, 2000; Siqueira *et al.*, 2001). To our knowledge, a quantitative real-time PCR assay has not yet been described, and fluorescent single-cell-specific assays are not available.

The motivation for the present study was our finding, in an ongoing investigation assessing selected microbiota in dental plaque from the gingival margins of Chinese patients with either necrotizing ulcerative gingivitis or gingivitis, that the former group had a significantly higher load of serologically detectable *P. intermedia*/*P. nigrescens* cells than the gingivitis group (data not shown). In light of the postulated different roles of the two species as periodontal pathogens, it seemed important to identify and quantify them differentially. Thus, the aim of the present study was to develop direct, quantitative, species-specific *P. intermedia* and *P. nigrescens* detection assays and to evaluate their performance in comparison with culture and indirect immunofluorescence (IF) applied, in parallel, to a set of subgingival plaque samples. Based on the available infrastructure and experience, we opted for fluorescent *in situ* hybridization (FISH) using DNA probes to species-specific 16S rRNA sequences to target the two species.

METHODS

Strains, dental plaque samples and cultivation. Strains of oral bacteria were grown anaerobically (85% N₂, 10% H₂, 5% CO₂) at 37 °C in fluid universal medium (Gmür &

Guggenheim, 1983) enriched with 5% horse serum and harvested after 12–24 h from the exponential phase of growth.

Subgingival plaque samples ($n = 39$) were obtained, with paper points (Gmür *et al.*, 1989), from periodontal pockets of 16 patients (14 female) with a mean age of 45.5 years (range 25–67 years). The subjects had been referred to our institute for microbial testing because of refractory chronic periodontitis (Armitage, 1999). The sampled pockets had a mean probing depth of 6.2 ± 1.3 mm (range 5–10 mm). Within 1 h of harvesting, samples were vortexed for 30 s at the maximum setting and split into different aliquots. Aliquots selected for FISH were frozen at -20 °C until used. Aliquots for IF and cultivation were processed immediately. Those reserved for cultures were serially diluted from 10^{-1} to 10^{-5} in 0.9% NaCl. A 50 µl aliquot from each dilution was distributed on Columbia blood agar base (Oxoid) supplemented with 5% haemolysed human blood using a spiral diluter (Spiral System), and plates were incubated anaerobically for 96 h at 37 °C. Colony-forming units (c.f.u.) were counted using a stereomicroscope, selectively assessing plates with an optimum concentration of colonies.

Cell fixation and preparation of multiwell slides. For FISH assays of cultured bacteria, strains were washed once in 0.9% NaCl, resuspended in sterile PBS at pH 7.4, and fixed (20 min, 4 °C) by mixing the cell suspension 1:10 (v/v) with 4% freshly prepared paraformaldehyde solution (Manz, 1999). Thereafter, the cells were pelleted by centrifugation at 10000 g, resuspended in 50% ethanol, and either directly spotted on precleaned 18- or 24-well microscope slides (Cel-Line Associates) or stored at -20 °C until used. Air-dried slides were dehydrated in 50, 80 and 100% ethanol (3 min each). For FISH assays of plaque samples, 10 µl diluted (1:5 or 1:10) plaque suspension per well was dropped onto 18- or 24-well slides, air-dried and then fixed immediately (20 min, 4 °C) by inserting the slide in a 50 ml centrifuge tube containing 4% paraformaldehyde. For IF assays, cultured bacteria or subgingival plaque samples were used to coat 18- or 24-well slides and fixed with methanol as described (Gmür *et al.*, 1989). Prior to the coating stage, subgingival plaque samples had been centrifuged at 10000 g for 5 min, resuspended for 3 min in 0.83% NH₄Cl to lyse erythrocytes, recentrifuged, resuspended in 0.9% NaCl/0.02% NaN₃/0.00025% cetyltrimethylammonium bromide and stored at -20 °C until used.

FISH. Custom-synthesized oligonucleotide probes, labelled at the 5'-ends with Cy3 or 6-FAM, were purchased from Interactiva. Probes were designed according to the criteria described by Manz (1999), using programs and rRNA sequence information from Ribosomal Database Project II (Maidak *et al.*, 1999) (<http://rdp.cme.msu.edu/html/>) and the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>). The sequences of the probes employed for *P. intermedia*, *P. nigrescens*, *Prevotella denticola* and *Prevotella pallens* are listed in Table 1. The probes were used at a final concentration of 5 ng µl⁻¹, except for competitor probes, which were used at 50 ng µl⁻¹. FISH was performed using a modification (Thurnheer *et al.*, 2001) of the method described by Manz (1999), except that the length of the hybridization period was set to 150 min. Optimum reactivity conditions for the probes with target and non-target strains were determined as described (Thurnheer *et al.*, 2001). The universal bacterial probe EUB338 (Amann *et al.*, 1995) served as a positive control. Fluorescence intensity was graded using an arbitrary five-step scale, where 0 (no fluorescence above background) and 1+ (very faint fluor-

Table 1. Probe sequences, target sites and optimum formamide concentrations for *in situ* hybridization

Probe	Target	Sequence (5'→3')*	Site†	Formamide (%)
EUB338	Bacteria	GCTGCCTCCCGTAGGAGT	338–355	5–40
Pint657	<i>P. intermedia</i>	TCCGCATACGTTGCGTGCA	657–675	40
Pint649	<i>P. intermedia</i>	CGTTGCGTGCACTCAAGTC	649–667	40
Pnig657	<i>P. nigrescens</i>	TCCGCCTGCGCTGCGTGTA	657–675	40
Ppal654	<i>P. pallens</i>	GCATACGTTGCGTG <u>I</u> ACTC	654–672	40
Ppal654comp	<i>P. intermedia</i>	GCATACGTTGCGTG <u>C</u> ACTC	654–672	40
Ppal186	<i>P. pallens</i>	CATATCTGATGCCATCAATG	186–204	10
Pden654	<i>P. denticola</i>	GCCGGCGTTGCGCGTACTC	654–672	40

* An underlined letter indicates a single mismatch between *P. intermedia* and *P. pallens* at position 658.

† Targeted 16S rRNA region (*E. coli* numbering; Brosius *et al.*, 1981).

escence) were considered negative signals, and 2+ (weak), 3+ (strong) and 4+ (brilliant fluorescence) were considered positive signals.

IF. *Porphyromonas gingivalis* and the *Prevotella intermedia*/*P. nigrescens* group of bacteria were labelled with mAbs 61BG1.3 (Gmür *et al.*, 1988) and 37BI6.1/39BI1.1.2 (Gmür & Guggenheim, 1983), respectively. Specific mAb binding by target cells was visualized (Gmür *et al.*, 1989) by using consecutive incubations with a mouse anti-rat κ -chain mAb (MARK-1; Bazin *et al.*, 1984) (an optional step used only in conjunction with the rat mAb detecting *P. intermedia*/*P. nigrescens*), a mixture of biotinylated goat anti-mouse IgG and IgM (both from Sigma) and avidin-fluorescein isothiocyanate (Sigma). The lower detection limit of the IF assay is 3×10^3 (or 0.003%) for a typical sample from a periodontal pocket containing approximately 10^8 bacteria (Gmür, 1995).

Microscopy. FISH- and IF-stained slides were read with an Olympus BX60 epifluorescence microscope [Olympus Optical (Schweiz)] equipped with phase-contrast, an HBO 103 W/2 mercury photo optic lamp (Osram) and Olympus filter sets U-MNIBA (6-FAM), U-MA41007 (Cy3) and BX-DFC5 (6-FAM/Cy3). The samples were evaluated for the number of fluorescent bacteria per ml of sample as described (Gmür & Guggenheim, 1994), monitoring either randomly selected fields that covered, in total, between 1/100 and 1/2 of a well or an entire well, depending on the density of positive cells. Colour micrographs were taken with a digital Olympus Camedia 3030 camera, transferred to a Macintosh G3 personal computer and processed using the standard software provided with the camera and PHOTOSHOP 6.0 (Adobe) without any qualitative changes to the raw images.

RESULTS

Specificity of oligonucleotide probes

In specificity tests with reference strains from various *Prevotella* species, probes Pnig657, Ppal186 and Pden654 showed the anticipated specificity for *P. nigrescens*, *P. pallens* and *P. denticola*, respectively (Table 2). Probe Pint657 identified all *P. intermedia* strains, but also hybridized to isolates of *P. pallens*. To avoid such cross-reactivity, a second probe to *P. intermedia* (Pint649) was

evaluated that had the single mismatch at nucleotide 658 shifted to the central region of the probe. Pint649 proved to be specific for *P. intermedia* and labelled all *P. intermedia* strains tested. For *P. pallens*, a second probe (Ppal654) was employed as well, but in this case with the aim of obtaining stronger fluorescence intensity. It revealed specificity for *P. pallens* if an unlabelled competitor sequence (Ppal654comp) was added during hybridization to prevent cross-reactivity with *P. intermedia* (Table 2). All probes directed against target sequences between nucleotides 645 and 675 led to very intensive fluorescence (3–4+), whereas fluorescence obtained with Ppal186 was much weaker and was graded as 2–3+. None of the probes hybridized to tested strains of other taxa (data not shown) or to labelled bacteria in plaque samples with atypical target-cell morphology.

Quantitative detection, using FISH, of target bacteria in subgingival plaque samples

Fig. 1 shows a field of a representative plaque sample stained for *P. intermedia* (with probe Pint649-Cy3; red) and *P. nigrescens* (with Pnig657-FAM; green). The two corresponding images were made in fluorescence mode using a dual filter set (Fig. 1a) and in phase-contrast mode (Fig. 1b). They demonstrate the main presence of both species in heterogeneous aggregates ranging in size from a few to several hundred cells. Planktonic *P. intermedia* and *P. nigrescens* bacteria accounted for only a very minor proportion (<5%) of the cells detected. Aggregates colonized by both species were not uncommon, but, in general, the cells of one species strongly outnumbered those of the other. Both *P. intermedia* and *P. nigrescens* presented in plaque as small cocci or very short rods. *P. denticola* cells were always rod-shaped and longer than *P. intermedia* or *P. nigrescens* cells (not shown).

Results from the application of probes Pint657 and Pnig657 to subgingival plaque samples are summarized

Table 2. Probe specificity assessed by FISH with various *Prevotella* species

Species	Reactivity* with probe:					
	Pint657	Pint649	Pnig657	Ppal654 + Ppal654 comp	Ppal186	Pden654
<i>P. buccae</i>	0/1	0/1	0/1	0/1	0/1	0/1
<i>P. corporis</i> †	0/2	0/2	0/2	0/2	0/1	0/2
<i>P. denticola</i> †	0/3	0/3	0/3	0/2	0/2	3/3
<i>P. intermedia</i> †	8/8	7/7	0/8	0/8	0/7	0/7
<i>P. loescheii</i> †	0/1	0/1	0/1	0/1	0/1	0/1
<i>P. melaninogenica</i>	0/2	0/2	0/2	0/2	0/2	0/2
<i>P. nigrescens</i> †	0/8	0/6	8/8	0/7	0/6	0/6
<i>P. oralis</i> †	0/1	0/1	0/1	0/1	0/1	0/1
<i>P. oris</i> †	0/1	0/1	0/1	0/1	0/1	0/1
<i>P. ouloris</i> †	0/1	0/1	0/1	0/1	0/1	0/1
<i>P. pallens</i> †	3/3	0/3	0/3	3/3	3/3	0/3
<i>P. veroralis</i>	0/1	0/1	0/1	0/1	0/1	0/1

* Number of positive strains versus total number of strains tested.

† Includes the type strain of the species.

Fig. 1. Subgingival plaque sample no. 36 stained by *in situ* hybridization with probes Pint649-Cy3 and Pnig657-FAM with specificity for *P. intermedia* (red) and *P. nigrescens* (green), respectively. Epifluorescence (a) and phase contrast (b) micrographs from one representative field are shown. Labelled cells are located almost exclusively within dense mixed aggregates. Often both species are present within an aggregate, but, in general, one species strongly outnumbers the other. Bar, 10 µm.

in Fig. 2(a), *P. intermedia* was detected in 27 of 39 (69%) and *P. nigrescens* in 26 of 39 (67%) samples. *P. pallens* was not detected in spite of using, with Pal186 and Ppal654 (plus Ppal654comp), two distinct probes. *P. denticola* occurred in 10 of 39 (26%) samples. Nine samples (23%) were negative for all monitored *Prevotella* species. Of the samples positive for *P. intermedia* or *P. nigrescens*, 77% harboured both organisms. The ratio between *P. intermedia* and *P. nigrescens* in double-positive samples varied considerably between different

samples and thus provided no hints as to possible synergistic or antagonistic effects between these two species. However, high levels of *P. nigrescens* ($>10^6$ cells per ml sample) always accompanied similarly high levels of *P. intermedia*, whereas the opposite was clearly not the case (Fig. 2a).

Although *P. pallens* was not detected with Ppal186 and Ppal654 (plus Ppal654comp) in the present collection of plaque samples, we opted to re-examine the presence of

Fig. 2. Bar chart showing the prevalence and number of *P. intermedia* and *P. nigrescens* cells in subgingival plaque samples analysed by FISH. (a) Data obtained using probes Pint657 and Pnig657 to identify *P. intermedia* and *P. nigrescens*, respectively. (b) Comparison of the enumeration of *P. intermedia* with probes Pint657 and Pint649, which, based on the available information on bacterial rRNA gene sequences from public data banks, should be specific for *P. intermedia*. Within the recognized target region, *P. intermedia* has at least two mismatches with any other known taxa, except *P. pallens*, which has one mismatch at position 658.

Fig. 3. Comparison of FISH with IF for the enumeration of *P. intermedia*/*P. nigrescens* in subgingival plaque. Since the IF assay with mAbs 37B16.1 and 39B11.1.2 cannot differentiate between the two species, the IF data are compared with the sum of *P. intermedia* and *P. nigrescens* cells detected by FISH.

P. intermedia with probe Pint649, which had not revealed cross-reactivity with *P. pallens* (Table 2). Because of the limited sample size, the analysis had to be restricted to 25 samples. Results are summarized in Fig. 2(b). Eight samples were negative with both probes; four more showed cell numbers of approximately 10^4 cells or less and do not qualify for comparisons because the counts were too close to the lower detection limit of the assay (approximately 3×10^3 cells per ml sample). Of the 13 remaining samples, eight displayed very good agreement between the cell numbers detected by Pint657 and Pint649. However, five samples, three of which (nos 26–28) were from the same patient, were positive with Pint657 and negative with Pint649; this strongly indicates that the former probe is detecting another unidentified organism of identical morphology to *P. intermedia*.

Comparison of FISH data with IF and culture data

P. intermedia and *P. nigrescens* enumerations by FISH and IF are compared in Fig. 3. Because the two species are not discriminated by the IF assay, the IF data had to be plotted against the sum of *P. intermedia* and *P. nigrescens* as detected with probes Pint657 and Pnig657. The results demonstrated a satisfactory correspondence ($r=0.710$) between the two assays, except for five samples that were positive by IF but negative by FISH.

For a comparison of FISH and IF with culture, Fig. 4 shows data from Columbia blood agar cultures plotted against the cell numbers of *P. intermedia* plus *P. nigrescens* as detected by both IF and FISH. Only *Porphyromonas gingivalis*-free samples (as determined by IF with mAb 61BG1.3; data not shown) were considered. It is evident that, with most samples, IF and

Fig. 4. Scattergram comparing FISH and IF with culture for the quantitative detection of *P. intermedia*/*P. nigrescens* in subgingival plaques. The 45° line indicates a theoretical ideal relationship between the black-pigmented c.f.u. and IF or FISH counts. The IF assay was performed with mAbs 37B16.1 and 39B11.1.2, and the FISH assay with probes Pint657 and Pnig657. All samples were free of *Porphyromonas gingivalis*. To facilitate reading, negative values were assigned a value of 2. ○, Pi+Pn by IF; ●, Pi+Pn by FISH.

FISH enumerations exceed c.f.u. counts considerably, on average by factors of 87 and 70, respectively. Again, this finding clearly reflects the main presence of these organisms in small, virtually undispersible, aggregates, described in Fig. 1.

DISCUSSION

The distribution and number of *P. intermedia* and *P. nigrescens* cells in 39 subgingival plaque samples from severe periodontitis patients were studied by applying a quantitative FISH assay directly to fixed specimens. Samples were further monitored for the recently described species *P. pallens* (Könönen *et al.*, 1998), which has a close genetic relationship to *P. intermedia*, and for *P. denticola*, which, like *P. intermedia*, was described as being associated with gingivitis and periodontitis (Wu *et al.*, 1992). The results from our FISH experiments showed that *P. intermedia* and *P. nigrescens* colonized the investigated subgingival plaque samples with virtually identical prevalence (69 versus 67%) and density (approx. 3×10^6 cells ml⁻¹). They closely resemble those reported by Mättö *et al.* (1996b). Previously, *P. intermedia*, but not *P. nigrescens* (the former *Bacteroides intermedius* genospecies II or homology group 8944), has been associated with periodontitis (Moore *et al.*, 1987; Dahlén *et al.*, 1990; Gharbia *et al.*, 1994; Mättö *et al.*, 1996b). However, this association was based as much on the absence or minimal presence of *P. intermedia* at healthy or weakly inflamed sites as on its abundance in deep periodontal pockets. Apparently, *P. nigrescens* colonizes more diverse niches of the oral cavity (Moore, 1987; Mättö *et al.*, 1996b; Baumgartner *et al.*, 1999). *P. pallens* was not observed in the present

study in spite of the application of two probes with specificity for distinct regions of the 16S rRNA of the species. It is not possible to compare our finding with previously reported data since earlier studies did not use techniques that would specifically recognize *P. pallens*. *P. denticola* was detected with a low prevalence (26%) and in slightly lower cell numbers than *P. intermedia* and *P. nigrescens*. It is the most frequently detected plaque organism of a group of *Prevotella* species that also includes *Prevotella melaninogenica* and *Prevotella loescheii*, and possibly could be confused with *P. intermedia*/*P. nigrescens* when screening blood agar cultures for black-pigmented colonies, although all three species frequently display weak red-brown or no pigmentation (Wu *et al.*, 1992). We used the probe for *P. denticola* in the present study to obtain an idea of the abundance of this micro-organism, which had until then been neglected in our analyses of plaques from periodontitis patients.

The rapid and accurate identification and enumeration of black-pigmented anaerobes from highly complex microbial communities such as subgingival plaque are difficult tasks. Many of the available techniques for differentiation between *P. intermedia* and *P. nigrescens* rely on culture as the initial step; black-pigmented colonies are selected for and counted, and then a variety of 'secondary' techniques are used to identify representative colonies (Dahlén *et al.*, 1990; Mättö *et al.*, 1996b; van Steenberghe *et al.*, 1997; Conrads *et al.*, 1997; Debelian *et al.*, 1997; Haraldsson & Holbrook, 1999; Robertson *et al.*, 1999). This approach has several disadvantages. First, it is expensive and time-consuming because of a lengthy incubation period. Second, data from this study (Fig. 4) demonstrate clearly that culture substantially underestimates the cell numbers of *P. intermedia* and *P. nigrescens* in subgingival plaque. This is not surprising in the light of the overwhelming association of both species with dense mixed bacterial aggregates (Fig. 1) that are not disrupted by brief sonication and lengthy vortexing (60 s). Similar evidence has been reported previously (Loesche *et al.*, 1992; Giertsen *et al.*, 2000); nevertheless, the problem that aggregation poses to quantitative culture analyses remains widely neglected. Techniques that do not require culturing and which differentiate between *P. intermedia* and *P. nigrescens* are available (various PCR-based assays, checkerboard DNA–DNA hybridization), but so far they have the disadvantage of yielding only qualitative (Ashimoto *et al.*, 1996; Riggio *et al.*, 1998; Stubbs *et al.*, 1999; Mullally *et al.*, 2000), or at best semi-quantitative, data (Socransky *et al.*, 1994; Tanner *et al.*, 1998). Clearly, there is a need for a rapid and objectively quantitative procedure for the direct quantification of the two species in clinical samples to investigate their postulated roles in oral health and disease in more detail. We feel that our FISH assay with new probes to species-specific stretches of 16S rRNA of *P. intermedia*, *P. nigrescens*, *P. pallens* and *P. denticola*, described in this work, provides such a tool. Unlike currently used IF assays, the FISH assay can differentiate between *P.*

intermedia, *P. nigrescens* and *P. pallens*. Like IF, it offers sensitive, qualitative and quantitative monitoring of single fluorescent cells in clinical samples, and has a lower detection limit of slightly more than 10^3 cells ml⁻¹ for samples containing approximately 10^8 bacteria. (Such cell densities require sample dilutions of 1:5 or 1:10 for the coating of 4 mm wells with 10 µl. If the minimum requirement for a positive score is set to at least three positive cells per well, it follows that the lower detection limit at a 1:10 dilution is 3×10^3 ml⁻¹; Gmür, 1995). Fluorescence assays for bacteria are always confronted with the possible presence of autofluorescence-emitting bacteria or of non-specifically fluorescent particles. In the present study, autofluorescence with the characteristic orange-red colour of the Cy3 label or the fluorescein-isothiocyanate-like colour of 6-FAM was not observed. Fluorescent particles were always present but could be recognized readily because of the extremely intensive fluorescence and the irregular shapes. A comparison of the performance of FISH and IF generally showed good correspondence (Fig. 3), in particular if one considers that increased data variability had to be expected because cells were distributed unevenly (in aggregates) in the wells of the microscope slide and only a part of the entire well was monitored. Interestingly, five samples were positive for *P. intermedia*/*P. nigrescens* by IF but not by FISH. The most likely explanation for this observation is that mAbs 37B16.1 and 39B11.1.2 could bind to another, so far unidentified, species. We had noted recently that both mAbs labelled strains of the newly described species *P. pallens* (unpublished observation), but, according to the FISH data with probes Ppal186 and Ppal654 (+Ppal654comp), *P. pallens* was not present in the samples studied.

In designing probes for the FISH assay described, we preferentially targeted the highly variable 5' half of helix 23 of the 16S rRNA with nucleotides 645–680 (*Escherichia coli* numbering; Brosius *et al.*, 1981). This region has yielded numerous bright oligonucleotides with specificity at genus or species level (Amann *et al.*, 1995; Fuchs *et al.*, 1998) (our unpublished data). All five probes from this study against this region were usable at high formamide concentrations and yielded very strong fluorescence signals. In contrast, a *P. intermedia*-specific probe targeted to nucleotides 620–638 gave completely negative results (data not shown). *P. intermedia* and *P. pallens* differ within the stretch from nucleotides 645–680 only at position 658. Thus, probes Pint657 and Pint649 (both 19-mer oligonucleotides) each have this single mismatch with *P. pallens*, which, however, is located either peripherally or centrally. Not unexpectedly (Manz, 1999), the peripheral mismatch location in probe Pint657 led to cross-reactivity with *P. pallens* at medium fluorescence intensities, whereas Pint649 with the mismatch in the centre of the probe proved to be *P. intermedia*-specific. Conversely, probe Ppal654 was only negative with *P. intermedia* if an unlabelled competitor probe was added in excess to prevent the hybridization of the fluorescent Ppal654 to *P. intermedia*. As all

samples of this study appeared to be *P. pallens*-free (no bacteria positive with Ppal186 or Ppal654/Ppal654comp could be detected), one would expect FISH with Pint657 and Pint649 to result in approximately the same number of fluorescent bacteria. This was indeed the case with most samples, which underlines the credibility of this quantitative assay. However, five exceptional samples, three from one patient, were noted. In these samples, Pint657 labelled between 10^5 and 10^7 positive bacteria that remained unlabelled by Pint649. Positive cells had the typical morphology of plaque-derived *P. intermedia*. The data suggest that Pint657 may detect (in addition to 'normal' *P. intermedia*) an unidentified variant or different taxa with at least one mismatch within the rRNA region (nucleotides 657–667) covered by both probes.

In summary, the results presented in this investigation show good discrimination between *P. intermedia* and *P. nigrescens* by oligonucleotide probes, and demonstrate the advantages of the application of a FISH assay specific for these species in comparison to both culture and IF.

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