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

Rudolf Gmürr and Thomas Thurnheer

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 $3 \times 10^3$ 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, $3.9 \times 10^6$ *P. intermedia*, $3.1 \times 10^6$ *P. nigrescens* and $5.6 \times 10^5$ *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 ($r = 0.71$). 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 Steenbergen et al., 1982; Johnson & Holdeman, 1983; Moncla et al., 1988; Fukushima et al., 1992), serological experiments (Gmürr & 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; Gmürr & 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...
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 (Gmü & Wyss, 1985; Shah & Gharbia, 1992; Gharbia et al., 1994; Frandsen et al., 1995; Dahlén et al., 1996; Debelian et al., 1996; Måttö 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 (Måttö 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 (Gmü & 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 (Gmü 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 (cfu) 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 (Cell 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 (Gmü 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% NaNO₂/0.00025% 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

<table>
<thead>
<tr>
<th>Probe</th>
<th>Target</th>
<th>Sequence (5′→3′)†</th>
<th>Site‡</th>
<th>Formamide (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB338</td>
<td>Bacteria</td>
<td>GCTGCTCCCGTAGGAGT</td>
<td>338–355</td>
<td>5–40</td>
</tr>
<tr>
<td>Pint657</td>
<td>P. intermedia</td>
<td>TCCGCTACGGTGGTGCA</td>
<td>657–675</td>
<td>40</td>
</tr>
<tr>
<td>Pint649</td>
<td>P. intermedia</td>
<td>GTCTCGGGTGACTCAGTC</td>
<td>649–667</td>
<td>40</td>
</tr>
<tr>
<td>Pnig657</td>
<td>P. nigrescens</td>
<td>TCCGCTCGCTGCTGTTGA</td>
<td>657–675</td>
<td>40</td>
</tr>
<tr>
<td>Ppal654</td>
<td>P. pallens</td>
<td>CATACGGTGTTAGTACTC</td>
<td>654–672</td>
<td>40</td>
</tr>
<tr>
<td>Ppal654comp</td>
<td>P. intermedia</td>
<td>GCATACGGTGTTAGTACTC</td>
<td>654–672</td>
<td>40</td>
</tr>
<tr>
<td>Ppal186</td>
<td>P. pallens</td>
<td>CATATCTGATGCCATCAATG</td>
<td>186–204</td>
<td>10</td>
</tr>
<tr>
<td>Pden654</td>
<td>P. denticola</td>
<td>GCCGCGGTTGCGCGTGACTC</td>
<td>654–672</td>
<td>40</td>
</tr>
</tbody>
</table>

*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).

Results from the application of probes Pint657 and Pnig657 identified all isolates of Porphyromonas gingivalis and Prevotella intermedia that showed the anticipated specificity for P. intermedia (Pint649) or P. nigrescens (Pnig657). 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

<table>
<thead>
<tr>
<th>Species</th>
<th>Pint657</th>
<th>Pint649</th>
<th>Pnig657</th>
<th>Ppal654 + Ppal654 comp</th>
<th>Ppal186</th>
<th>Pden654</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. buccae</em></td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td><em>P. corporis</em>†</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/1</td>
<td>0/2</td>
</tr>
<tr>
<td><em>P. denticola</em>†</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/2</td>
<td>0/2</td>
<td>3/3</td>
</tr>
<tr>
<td><em>P. intermedia</em>†</td>
<td>8/8</td>
<td>7/7</td>
<td>0/8</td>
<td>0/8</td>
<td>0/7</td>
<td>0/7</td>
</tr>
<tr>
<td><em>P. loeschei</em>†</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td><em>P. melaninogenica</em></td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td><em>P. nigrescens</em>†</td>
<td>0/8</td>
<td>0/6</td>
<td>8/8</td>
<td>0/7</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td><em>P. oralis</em>†</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td><em>P. oris</em>†</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td><em>P. ouloris</em>†</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td><em>P. pallens</em>†</td>
<td>3/3</td>
<td>0/3</td>
<td>0/3</td>
<td>3/3</td>
<td>3/3</td>
<td>0/3</td>
</tr>
<tr>
<td><em>P. veroralis</em></td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
</tr>
</tbody>
</table>

* Number of positive strains versus total number of strains tested.
† Includes the type strain of the species.

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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.

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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* (>10⁶ 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
Oral *P. intermedia* and *P. nigrescens* enumeration

*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 $3 \times 10^3$ 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...
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 undisruptible, 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. $3 \times 10^6$ cells ml$^{-1}$). They closely resemble those reported by Mätto 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; Dahlen et al., 1990; Gharbia et al., 1994; Mätto 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; Mätto 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 (Dahlen et al., 1990; Mätto et al., 1996b; van Steenbergen 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 $10^5$ 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 $3 \times 10^5$ ml$^{-1}$; Gmüör, 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 37Bl6.1 and 39Bl1.2 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 Pint648 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 $10^6$ 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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