Direct quantitative differentiation between *Preventella intermedia* and *Preventella 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 *Preventella intermedia* and *Preventella 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*, *Preventella pallens* and *Preventella 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 *Preventella* 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 ($r = 0.711$). 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 *Preventella*, some of which may have clinical significance. The two most prevalent species of the group are *Preventella intermedia* and *Preventella 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 (Gmürr & Wyss, 1985; Shah & Gharbia, 1992; Gharbia et al., 1994; Frandsen et al., 1995; Dahlén et al., 1996; Debelian et al., 1996; Mätö 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 (Mätö et al., 1996a, b; Conrad 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; Mullanly 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 in dental plaque from the gingival margins of Chinese patients with either necrotizing ulcerative gingivitis or in dental plaque from the gingival margins of Chinese patients with either necrotizing ulcerative gingivitis or...
reactivity, a second probe to

Porphyromonas gingivalis and

Prevotella pallens

and

P. nigrescens

qualitative changes to the raw images.

with the camera and

Camedia 3030 camera, transferred to a Macintosh G3 personal

Colour micrographs were taken with a digital Olympus

Olympus BX60 epifluorescence microscope (Olympus Optical

fluorescence) were considered negative signals, and 2 + (weak),

3 + (strong) and 4 + (brilliant fluorescence) were considered

positive signals.

Porphyromonas gingivalis and the Prevotella intermedia/

Porphyromonas gingivalis and the Prevotella intermedia/

Prevotella pallens group of bacteria were labelled with mAbs

61BG1.3 (Gmürr et al., 1988) and 37BL6.1/39B11.1.2 (Gmürr

& Guggenheim, 1983), respectively. Specific mAb binding by

target cells was visualized (Gmürr et al., 1989) by using

consecutive incubations with a mouse anti-rat k-chain mAb

(MARK-1; Bazin et al., 1984) (an optional step used only

in conjunction with the rat mAb detecting

Prevotella intermedia/

Prevotella intermedia/Prevotella nigrescens), a mixture of biotinylated goat anti-mouse IgG and IgM (both from Sigma) and avidin-fluorescein isothio-

cyanate (Sigma). The lower detection limit of the IF assay is

3 x 10^9 (or 0.003 %) for a typical sample from a periodontal

pocket containing approximately 10^10 bacteria (Gmürr, 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 (Gmürr &

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. inter-

media 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. inter-

media (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.

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>GCTGCTCCCCTAGGAGT</td>
<td>338–355</td>
<td>5–40</td>
</tr>
<tr>
<td>Pint657</td>
<td>P. intermedia</td>
<td>TCCGCATACGTTGCGTGCA</td>
<td>657–675</td>
<td>40</td>
</tr>
<tr>
<td>Pint649</td>
<td>P. intermedia</td>
<td>GCTTGCCTGGACTCAAGTC</td>
<td>649–667</td>
<td>40</td>
</tr>
<tr>
<td>Pnig657</td>
<td>P. nigrescens</td>
<td>TCCGCCTGCGCTGCGTGTA</td>
<td>657–675</td>
<td>40</td>
</tr>
<tr>
<td>Ppal654</td>
<td>P. pallens</td>
<td>GCATACGTTGCGTGACTC</td>
<td>654–672</td>
<td>40</td>
</tr>
<tr>
<td>Ppal654comp</td>
<td>P. intermedia</td>
<td>GCATACGTTGCGTGACTC</td>
<td>654–672</td>
<td>40</td>
</tr>
<tr>
<td>Ppal186</td>
<td>P. pallens</td>
<td>CATCTAGATGCCATCAATG</td>
<td>186–204</td>
<td>10</td>
</tr>
<tr>
<td>Pden654</td>
<td>P. denticola</td>
<td>GCCGGCGTGGCGGTACCT</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).
Table 2. Probe specificity assessed by FISH with various *Prevotella* species

<table>
<thead>
<tr>
<th>Species</th>
<th>Reactivity* with probe:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pint657</td>
</tr>
<tr>
<td><em>P. buccae</em></td>
<td>0/1</td>
</tr>
<tr>
<td><em>P. corporis</em>†</td>
<td>0/2</td>
</tr>
<tr>
<td><em>P. denticola</em>†</td>
<td>0/3</td>
</tr>
<tr>
<td><em>P. intermedia</em>†</td>
<td>8/8</td>
</tr>
<tr>
<td><em>P. loescheii</em>†</td>
<td>0/1</td>
</tr>
<tr>
<td><em>P. melaninogenica</em></td>
<td>0/2</td>
</tr>
<tr>
<td><em>P. nigrecens</em>†</td>
<td>0/8</td>
</tr>
<tr>
<td><em>P. oralis</em>†</td>
<td>0/1</td>
</tr>
<tr>
<td><em>P. oris</em>†</td>
<td>0/1</td>
</tr>
<tr>
<td><em>P. oulor</em>†</td>
<td>0/1</td>
</tr>
<tr>
<td><em>P. pallens</em>†</td>
<td>3/3</td>
</tr>
<tr>
<td><em>P. veroralis</em></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.

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. nigrecens* (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. nigrecens* 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. nigrecens*, 77% harboured both organisms. The ratio between *P. intermedia* and *P. nigrecens* 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. nigrecens* (>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 undisturbable, 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. $3 \times 10^8$ 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 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 10^6 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 x 10⁷ ml⁻¹; Gmuir, 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 39B11.2 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 10^3 and 10^5 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.

ACKNOWLEDGEMENTS

We thank Martin Gander and Yvonne Helweg for excellent technical assistance.

REFERENCES


Shah, H. N. & Gharbia, S. E. (1992). Biochemical and chemical studies on strains designated *Prevotella intermedia* and proposal


\textit{Received 28 September 2001; revised 7 January 2002; accepted 28 January 2002.}