Optimized Oligonucleotides for the Differentiation of *Prevotella intermedia* and *Prevotella nigrescens*

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Abstract

The gram-negative, anaerobic bacterium *Prevotella intermedia* sensu stricto plays an important role in the progression of periodontitis, whereas the etiological role of the closely related but phenotypically indistinguishable species *Prevotella nigrescens* is controversial. To differentiate between these species properly, 16S rDNA/RNA directed, computer-optimized

oligonucleotides were designed and tested with 26 *P. intermedia*, 26 *P. nigrescens* and a number of closely and more distantly related strains. The oligonucleotides were used as primers in a polymerase chain reaction (PCR) and could be demonstrated to be species specific with a detection limit of 50 bacterial cells, which could also be detected when diluted $1:10^5$ with different plaque bacteria. In addition, the described oligonucleotides were digoxigenin- labeled at the 3'end and used as DNA probes in a dot blot hybridization assay. This assay, although slightly less sensitive than the PCR-based method, gave species-specific reactions, and also allowed (semi-)quantification of bacterial cells in clinical specimens.

Introduction

The "black-pigmented bacteroides" have undergone major taxonomic changes at both the generic and species level within the last decade (13, 15). Whereas assacharolytic strains have been placed in the genus *Porphyromonas*, strains that ferment carbohydrates are combined in the genus *Prevotella*. Among this group are species involved in the formation and progression of oral and odontogenic infections like periodontitis, endodontitis or anaerobic lung empyema (1, 8). In particular, the moderately saccharolytic pigmented species *Prevotella intermedia* and *Prevotella nigrescens* (formerly known as *Prevotella* [*Bacteroides*] *intermedia* genotype 1 and 2 [6, 16, 17]) seem to play an important etiological role in these diseases (8, 19, 21). It is increasingly reported that *P. intermedia* is associated with periodontitis, whereas *P. nigrescens* is a natural commensal of the gingival sulcus and the supragingival plaque (3, 7-11), but evidence against this theory still exists (12, 20). In addition, *P. nigrescens* seems to be more frequently isolated from endodontic infections (8). However, the true roles of these closely related organisms in health and disease remain unclear, and definitive studies are dependent on good techniques to distinguish between them in both, pure cultures and clinical specimens.

For these reasons and because of the availability of new programs and updated databases for optimizing DNA-probes and primers, we constructed two new 16S rDNA-directed oligonucleotides and tested them with a panel of *P. intermedia*, *P. nigrescens*-strains as well as closely and more distantly related bacterial species.

Material and methods

Bacterial strains

The complete DNA/RNA was isolated from both reference strains and molecular biological (ribotyping, enzyme electrophoresis) characterized clinical isolates, representing the gram-negative anaerobes: *P. intermedia* (ATCC 25611, L1440, LR22, L583, LR15, LR78, LK71, F1/12, F2/26, F7/24, F11/17, F11/21, F16/16, F23/26, F28/11, F30/33, F30/41, A735, A738, Hg404, Hg1103, Hg1678, Hg1269, Hg1674, MH3, MH6), *P. nigrescens* (ATCC 33563, LR53, LR35, L621, LR100, LR119, LK65, LK 80, L610, LR20, F3/44, F13/24, F22/15, F39/33, A4, A567, A785, Hg403, Hg1272, Hg1681, Hg653, MH1, MH5, MUI 21, ACJ5, ACJ8), *Prevotella corporis* (A350, A363, A417, A646, A648, A709, A818), *Porphyromonas gingivalis* (ATCC 33277, W83, 381), *Porphyromonas asaccharolytica* ATCC 25260, *Actinobacillus actinomycetemcomitans* ATCC 33384, *Capnocytophaga ochracea* ATCC 33596, *Capnocytophaga sputigena* ATCC 33612, *Capnocytophaga gingivalis* ATCC 33624, *Fusobacterium nucleatum* ATCC 25586, and *Eikenella corrodens* ATCC 23834. The origin of strains was: L-strains: Dr. H. Shah, Eastman Dental Institute, London, UK; F-strains: Dr. K. Pelz, Institut für Mikrobiologie und Hygiene, Freiburg, Germany; AC-strains: Institut für Mikrobiologie, Aachen, Germany; A-strains: Dr. I. Mitchelmore, St. Bartholomew's Hospital Medical College, London, UK; Hg-strains: Dr. T.J.M. Van Steenbergen, Academic Centre for Dentistry, Amsterdam, The Netherlands; MUI-strain: Dr. D. Drucker, Dept. of Cell &

Structural Biology, Manchester, UK; MH-strains: Prof. M. Haapasaalo, Dept. of Cariology, Helsinki, Finland.

Oligonucleotide design and labeling

The complete 16S rRNA sequences of *P. intermedia* and *P. nigrescens* (accession numbers: L16468 [GenBank], X73965 [EMBL], L16471, L16479, and X73963) were aligned using the program PC/Gene (version 6.85, IntelliGenetics, Mountainview, California, USA). Regions for an optimized species specific annealing of DNA-probes and primers were selected with the aid of the program OLIGO (version 5.0, National Biosciences, Plymouth, Minnesota, USA). Figure 1 shows the selected region between bases 600 and 650. The deduced complementary oligonucleotides (Pi: 5'-GTTGCGTGCACTCAAGTCCGCC-3'; T_d= 78.5°C for the detection of *P. intermedia* and Pn: 5'-CCTGCGCTGCGTGTACTCAAGTCTG-3'; T_d= 76.6°C for the detection of *P. nigrescens*) were analyzed by the same program and found to be non self-complementary. In addition their specificity was tested with the program CHECK_PROBE (Ribosomal Database Project [RDP], Urbana, Illinois, USA) by comparing the sequences with the over 3000 16S rDNA data available from the RDP database. The oligonucleotides were synthesized on a Beckman Oligo 1000 DNA synthesizer (Beckman, Munich, Germany). After deprotection by incubation for 5 min in a 32%ammonia solution, the oligonucleotides were desalted using Sephadex G25 columns (Pharmacia, Bromme, Sweden). Afterwards, they could be used either labelled with digoxigenin as DNA probes in a dot blot hybridization assay, or directly as a reversed primer in a specific PCR reaction.

Fig. 1. Region on the 16S rDNA (bp 600-650) of *Prevotella intermedia* (ATCC 25611) and *Prevotella nigrescens* (ATCC 33563) from which the specific oligonucleotides (Pi, Pn) were deduced.

Nucleic acids techniques

DNA/RNA-Isolation, dot-blot hybridization and detection by using the Boehringer digoxigenin/chemiluminescences assay was performed by the corresponding Boehringer protocols and as previously described in detail (2). Briefly, nucleic acids (DNA and RNA) were isolated from the reference strains (defined cell concentrations) and plaque samples after lysozyme, SDS/proteinase K treatment by a standard phenol/chloroform extraction, blotted on to a nylon membrane (Biodyne B, PALL, Portsmouth, UK) and immobilized by UV-crosslinking. The stringent hybridization conditions were: 5 pmol digoxigenin-labeled Pi-probe or Pn-probe in a hybridization solution containing 6x SSC, 5x Denhardt's , 0.5% SDS, and 1µg/ml denaturated Salmonsperm-DNA at 55-70°C (Pi) or 60-70°C (Pn), respectively, overnight or for at least 6 h.

PCR amplification was carried out in a volume of 100 µl containing 1x PCR buffer and 2 units of Taq-polymerase (Boehringer Mannheim, Germany), 0.2 mM of each dNTP (Boehringer-Mannheim, Germany), 5 pmol universal 16S rDNA forward primer (pA: 5'-AGAGTTTGATCCTGGCTCAG-3'), 5 pmol of the specific 16S rDNA directed reversed primer, as described in the previous paragraph, and 1 µl (about 500 ng) of the template nucleic acid. The PCR reaction mixture was overlaid with mineral oil and amplified using a thermocycler (Trio-Thermoblock, Biometra Goettingen, Germany). The amplification was performed using the following temperature profile and 30 cycles: denaturation: 94^{o}C for 1 min., stringent annealing temperature 60-70°C (Pi-reversed primer) or 70-75°C (Pn-reversed primer) for 1 min, 72 ^{o}C for 2.5 min. Amplification products (aliquots of 10 µl) were separated electrophoretically on a 1 % agarose gel (Seaplaque: Biozym, Hameln, Germany) in 1x TPE (80 mM Tris-phosphate, 2 mM EDTA, pH 7.5) using a submarine agarose gel unit (Mini-D, cti, Idstein, Germany) at 35 V for 2.5 h. The gels were incubated in ethidium-bromide (2 mg/l TPE) for 5-15 min.

Results and Discussion

Both newly designed oligonucleotides for the differentiation of *P. intermedia* and *P. nigrescens* proved to be specific for all strains tested in the dot blot hybridization assay and the PCR reaction.

Figure 2 shows a representative dot blot hybridization result using the type strains and 16 reference strains. The detection limit of this assay was 10^3 cells and the optimal hybridization temperature was 65°C for both probes. Using higher temperatures (66-70°C) increased the background of the chemiluminescence-detection system, but the reaction was specific for a range of template-concentrations between 50 pg and 1 µg. Using lower temperatures (55-64°C), the deduced detection signals led to an increase in cross-hybridization but only when template-concentrations $\underline{\geq}$10 ng were blotted. Diluting the *P. intermedia* or the *P. nigrescens* cells 1:10^5 with other oral gram-negative bacteria or subgingival plaque revealed no reduction of specificity and sensitivity. We applicated decreasing amounts of nucleic acids (5- 0.05 ng) corresponding to 10^5-10^3 cells of the type strains on the blot (figure 2, lanes A and B [reference dots]). By comparing the signal intensity of the sample dots with the reference dots (10^5-10^3 bacteria), a (semi-)quantification of the bacteria in an individual sample (e.g. subgingival plaque) can be made, as we described previously (2).

Figure 3 demonstrates a representative PCR result obtained with the type strains of both species using increasing annealing temperatures from 40 to 75°C. As suspected, by using the specific oligonucleotides (Pi, Pn) as reversed primers in combination with a highly conserved forward primer (pA), bands of nearly 660 bp appeared. The reaction for *P. intermedia* was specific in a wide range of annealing temperatures (60-70°C) whereas *the P. nigrescens* PCR should be performed at a temperature between 70 and 75°C.

Under these conditions all of the 52 *P. intermedia* or *P. nigrescens* strains analyzed revealed bands whereas all of the other strains listed above remained negative. The detection limit of this assay was calculated to be 50 cells (about 2.5 pg total nucleic acids). Once more, diluting the specific nucleic acids $1:10^5$ (2.5 pg to 0.25 µg) with the competitor DNA (*P. corporis*, *P. gingivalis*, subgingival plaque) did not reduce the specificity and the sensitivity of the PCR reaction.

Because of the limited value of physiological tests (e. g. peptidase and lipase activities) to separate between *P. intermedia* and *P. nigrescens* (16, 17), a number of molecular biological tests including multilocus enzyme electrophoresis (17), monoclonal antibodies (4) genomic DNA-DNA hybridization (6) and ribotyping (14) have already been applied. The application of these techniques in clinical studies are limited because they rely on using pure cultures. It should be possible to use monoclonal antibodies directly on clinical specimens, but unfortunately the monoclonal antibody which differentiates *P. intermedia* and *P. nigrescens* shows low sensitivity in immunofluorescence studies (9).

In addition, Dix et al. (5) designed 7 species-specific 16S rRNA/DNA directed oligonucleotide sequences (1Bi-1, 2, 3, 5, 6 [*P. intermedia*] and 2Bi-1, 2 [*P. nigrescens*]) to differentiate between these species. Shah et al. (18), in agreement with our findings, demonstrated that most of these probes lacked specificity or were constructed from hypervariable regions. Nevertheless, probes 1Bi-1 and 2Bi-2 were specific under conditions of high stringency but with a low tolerance to changes in the hybridization conditions, particularly a reduction of the hybridization temperature. In addition, a computerized analysis of these oligonucleotides (program OLIGO) revealed internal loops with a melting point between 44 and 56°C which could cause a reduction in the probe or primer concentration as assessed by dot-blot or PCR assays. The oligonucleotides designed in the present study were optimized for their application in a hybridization or PCR assay, making them useful for

screening clinical samples, such as subgingival plaque samples, for the qualitative distribution of *P. intermedia* and *P. nigrescens*.

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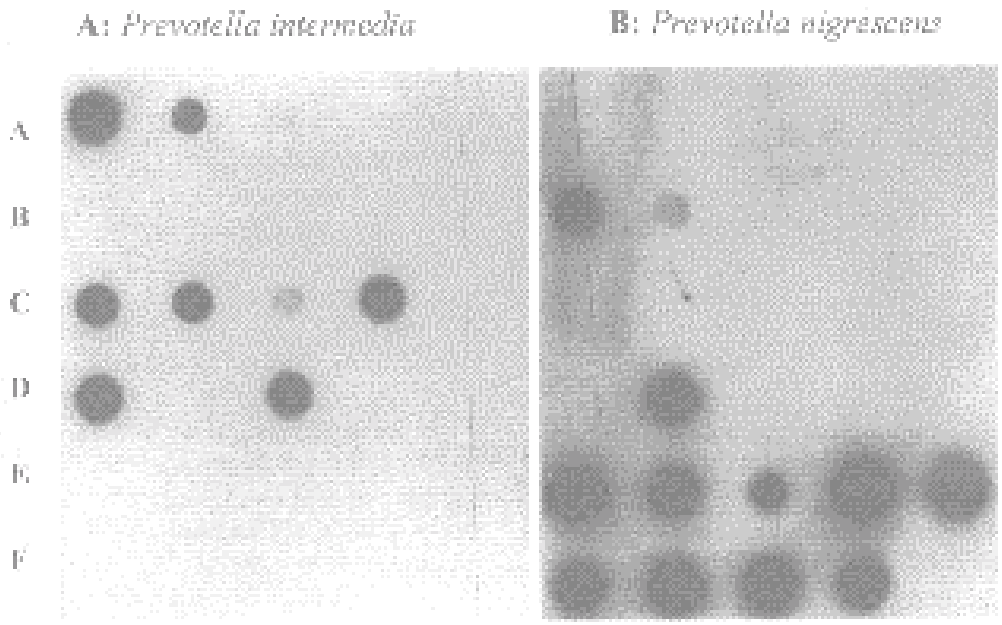

Fig. 2. Representative dot blot hybridization for the detection of *P. intermedia* and *P. nigrescens*: lane **A**: ATCC 25611 [10^5, 10^4, and 10^3 cells], lane **B**: ATCC 33563 [10^5, 10^4,

and 10^3] cells, lane **C**: L1440, LR22, L583, LR15, lane **D**: LR78, LR53, LK71, lane **E**: LR35, L621, LR100, LR119, LK65, and lane **F**: LK 80, L610, LR20, ACJ5. The cell concentrations in row C to F is between 10^3 [strain L583] and 10^6 [strain LR119]). In **blot A** the Pi-probe and in **blot B** the Pn-probe was used. No cross-reactions appeared by using 65°C as the hybridization temperature.

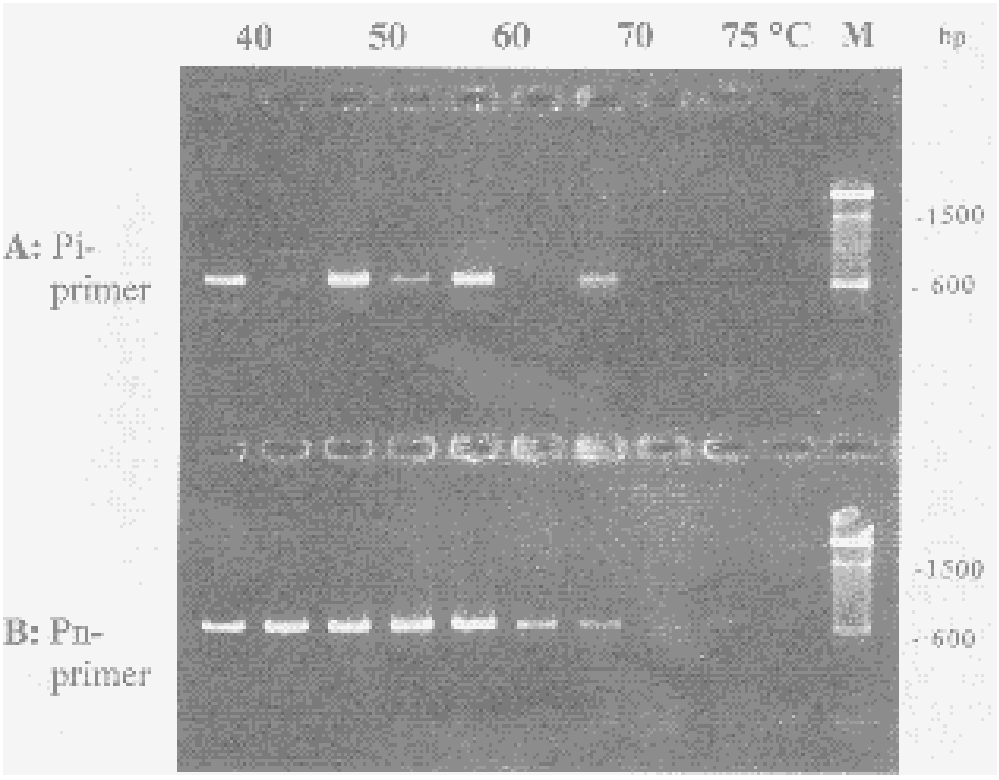

Fig. 3. Evaluation of the optimal annealing temperature (T_{opt}) for the *P. intermedia*/*P. nigrescens* specific PCR. Oligonucleotides Pi and Pn were used as reversed primers in combination with the universal 16S rDNA directed forward primer. For each temperature two PCRs with the (1.) specific and the (2.) competitor template were performed (**row A**: 1. ATCC 25611, 2. ATCC 33563; **row B**: 1. ATCC 33563, ATCC 25611). The *P. intermedia*-

PCR is specific in a temperature-range between 60-70°C, the *P. nigrescens*-PCR between 70 and 75°C.

Marker, 100 bp ladder (GIBCO BRL).

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