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Original

Primer design for the identification and detection of black-pigmented anaerobe rods using multiplex PCR

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Abstract

Black-pigmented anaerobe rods (BPAR) including genera *Porphyromonas* and *Prevotella* are regarded as a key factor in human oral infections. The purpose of the present study was to design primers to identify and detect the representative BPAR using multiplex Polymerase chain reaction (PCR). PCR primers were designed based on partial sequences of the 16S rDNA genes of the representative BPAR. These primers were able to distinguish each *Prevotella* and *Porphyromonas* species and did not display cross-reactivity with representative *Prevotella* species other than BPAR. Moreover, we developed a multiplex PCR method with the ability to identify and differentiate the representative BPAR, i.e. five *Prevotella* species and five *Porphyromonas* species, using only two PCR tubes per sample.

Key words : Black-pigmented anaerobe rods, *Prevotella*, *Porphyromonas*, Multiplex PCR

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1. Introduction

Black-pigmented anaerobe rods (BPAR) including genera *Porphyromonas* and *Prevotella* are regarded as a key factor in human oral infections of periodontic and endodontic origin[1-3]. BPAR are Gram-negative, strictly anaerobic, nonmotile, nonsporeforming, pleomorphic rods, and create glossy black color on blood agar, and give off foul odors. BPAR have undergone major taxonomic revisions at both the generic and species levels within three decades. Strains that ferment carbohydrates have been assigned to the genus *Prevotella*[4], while most of the strains that are assaccharolytic have been placed in the genus *Porphyromonas*[5]. The genus *Prevotella* currently comprises 50 species and *Porphyromonas* comprises 20 species (http://www.bacterio.net). These organisms require hemin and menadione for the growths. Among BPAR, *Porphyromonas gingivalis* is well known as a putative etiologic pathogen in the progression of periodontal disease, because this bacterium particularly has been associated with periodontally active diseased sites and loss of periodontal attachment[6-8]. BPAR other than *P. gingivalis* are also isolated from polymicrobial oral infections[1-3]. Therefore, the detection of individual species of bacteria in each patient should help to predict patient prognoses and to

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provide treatment strategies. However, as the detection of anaerobic bacteria can be problematic because of their specific growth requirements, certain technical barriers need to be overcome before information about such as bacterial species can be provided for routine dental clinics.

Recent advances in molecular biology have enabled the identification of specific bacteria in large numbers of periodontitis subjects. The polymerase chain reaction (PCR) is a commonly used technique and is used for the identification of periodontopathic bacteria in many laboratories. PCR rapidly and specifically identifies the presence of bacteria, and has been used to investigate the involvement of certain bacteria in the pathology of periodontitis in comparison with previous data obtained by bacterial culture[9].

To develop a more rapid and easier-to-use system for clinicians than conventional PCR, we developed a multiplex PCR system for identifying and detecting five *Porphyromonas* species (i.e., *P. gingivalis*, *Porphyromonas uenonis*, *Porphyromonas endodontalis*, *Porphyromonas asaccharolyticus*, *Porphyromonas bennonis*) and five *Prevotella* species (*Prevotella melaninogenica*, *Prevotella loescheii*, *Prevotella nigrescens*, *Prevotella denticola*, *Prevotella intermedia*). Their distribution in different oral sites and their role in common oral afflictions, particularly periodontal diseases, remains equivocal. Therefore, the accurate identification and detection of *Porphyromonas* and *Prevotella* species are required in order to clarify their role in oral ecology and dental diseases. Although conventional biochemical assays are used to identify *Porphyromonas* and *Prevotella* species, they are often imprecise due to the phenotypic variations displayed by these bacteria. Although a sequence analysis of several target genes is the most reliable method, it is expensive, laborious, and time-consuming. Thus, a simple and more reliable assay for identifying *Porphyromonas* and *Prevotella* species is desired. The purpose of the present study was to design primers for the identification and detection of BPAR, i.e. *Porphyromonas* and *Prevotella* species, using multiplex PCR.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The following bacterial strains were used in the present study: *P. uenonis* JCM 13868, *P. endodontalis* ATCC 35406, *P. gingivalis* ATCC 33277, *P. asaccharolyticus* JCM 6326, *P. bennonis* JCM 16335, *P. melaninogenica* JCM 6325, *P. loescheii* JCM 8530, *P. intermedia* ATCC 25611, *P. nigrescens* JCM 6322, *P. denticola* JCM 13449, *Prevotella oralis* ATCC17929, *Prevotella heparinolytica* ATCC 12102, *Prevotella buccae* JCM 3067, *Prevotella oris* ATCC 14266, and *Prevotella veroralis* ATCC 33449. These strains were maintained by cultivating them on anaerobic blood agar (CDC), which has a Tryptic soy agar (Becton, Dickinson and Co., Sparks, MD, USA) base supplemented with vitamin K_1 (10 μg/ml), hemin (5 μg/ml), L-cysteine (800 μg/ml), 0.5% yeast extract, and 5% sheep blood. These organisms were cultured at 37℃ for 48 h in an anaerobic jar with a gas pack system (AnaeroPack®, Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan).

2.2. Design of species-specific primers for five specie

The 16S rRNA sequences of *P. gingivalis* (accession no. AB035459), *P. endodontails* (AY253728), *P. asaccharolyticus* (L16490), *P. bennonis* (EU414673), *P. uenonis* (AY570514), *P. intermedia* (X73965), *P. melaninogenica* (AY323525), *P. denticola* (AY323524), *P. loescheii* (AY836508), and *P. nigrescens* (X73963) were obtained from the DNA Data Bank of Japan (DDBJ; Mishima, Japan), and a multiple sequence alignment analysis was performed with the CLUSTAL W program; i.e., the 16S rRNA sequences of ten BPAR were aligned and analyzed. Homology among the primers selected for ten BPAR species and their respective 16S rRNA sequences was confirmed by a BLAST search.

2.3. Development of a multiplex PCR method using designed primers

In the present study, ten BPAR were divided into two genus groups for a multiplex PCR

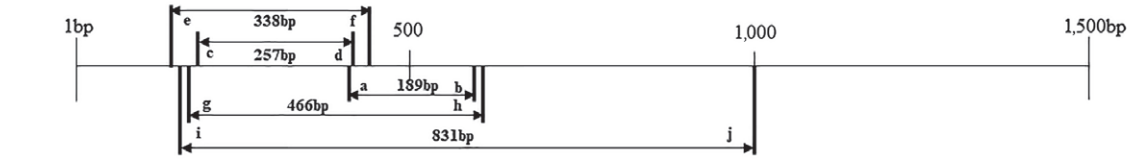
method. *Porphyromonas* group consisted of *P. gingivalis*, *P. endodontails*, *P. asaccharolyticus*, *P. bennonis*, *P. uenonis*, and *Prevotella* group consisted of *P. intermedia*, *P. melaninogenica*, *P. denticola*, *P. loescheii*, and *P. nigrescens*.

Bacterial cells were cultured in Tryptic soy broth (Becton, Dickinson and Co., Sparks, MD, USA) supplemented with vitamin K_1 (10 μg/ml), hemin (5 μg/ml), and 0.5% yeast extract for 24 h, and 1-ml samples were then collected in microcentrifuge tubes and resuspended at a density of 1.0 McFarland standard (approximately 10^7 colony-forming units (CFU)/ml) in 1 ml of sterile distilled water. A total of 3.6 μl of the suspension was then used as a PCR template. The detection limit of PCR was assessed by serially diluting known numbers of bacterial cells in sterile distilled water and then subjecting each suspension to PCR. The multiplex PCR mixture contained 0.2 μM of each primer of each group, 10 μl of 2×MightyAmp Buffer Ver.3 (Takara Bio Inc., Shiga, Japan), 0.4 μl of MightyAmp DNA Polymerase (Takara), and 5 μl of the template in a final volume of 20 μl. PCR reactions were performed in a DNA thermal cycler (Applied Biosystems 2720 Thermal Cycler; Applied Biosystems, CA, USA). PCR conditions included an initial denaturation step at 98℃ for 2 min, followed by 30 cycles consisting of 98℃ for 10 s and 68℃ for 1 min. PCR products were analyzed by 2.0% agarose gel electrophoresis before being visualized by electrophoresis in 1×Tris-borate-EDTA on a 2% agarose gel stained with ethidium bromide. A 100 bp DNA ladder (Takara Biomed, Shiga, Japan) was used as a molecular size marker.

3. Results

3.1. Primer design

Twenty specific primers covering the upstream regions of the 16S rDNA sequences of BPAR were designed in the present study (Fig. 1, 2). The specific forward primers of *Porphyromonas* group were designated as PUF for *P. uenonis*, PEF for *P. endodontalis*, PGF for *P. gingivalis*, PAF for *P. asaccharolyticus*, and PBF for *P.*



a: Forward primer for *Porphyromonas uenonis*
PUF : CGTCTACGTGTAGACGTTT 438-456bp

P. uenonis	CGTCTACGTG-TAGACGTT-T
P. endodontalis	CAGCTACGTG-TAGCTGAGAT
P. gingivalis	GCGATACGAG-TA-TTGCATT
P. asaccharolyticus	CACCTACGTG-TAGGTNTT-T
P. bennonis	CTTCCACGTGATGGGAGTT-T

b: Reverse primer for *Porphyromonas uenonis*
PUR : CTAGAGAGTTTCAAAGGCAAGA 626-605bp

P. uenonis	CTAGAGAGTTTCAAAGGCAAGA
P. endodontalis	CATATCAGTTTCAACGGCAAGA
P. gingivalis	CCCGGCAGTTTCAACGGCAGGC
P. asaccharolyticus	CTAGAGAGTTTCAAAGGCAAGA
P. bennonis	TCTTGCAGTTTCAACGGCAAGG

c: Reverse primer for *Porphyromonas endodontalis*
PEF : TGATTACAGATGGGCATG 197-214bp

P. uenonis	CGCTAAGAGATGGGCATG
P. endodontalis	TGATTACAGATGGGCATG
P. gingivalis	AGCTGTAAGATAGGCATG
P. asaccharolyticus	CGCTAAGAGATGGGCCTG
P. bennonis	CGCTGTGAGATAGGCATG

d: Reverse primer for *Porphyromonas endodontalis*
PER : TCTCAGCTACACGTAGCTGC 453-434bp

P. uenonis	-AACGNCTA-CACGTAGACGA
P. endodontalis	TCTCAGCTA-CACGTAGCTGC
P. gingivalis	ATGCAA-TA-CTCGTATCGCC
P. asaccharolyticus	-AANACCTA-CACGTAGGTGA
P. bennonis	-AACTCCCATCACGTGGAAGT

e: Forward primer for *Porphyromonas gingivalis*
PGF : ACAGAGGGGGATAACCCGTT 139-158bp

P. uenonis	TTAGTGGTGAATAACTCGAT
P. endodontalis	TAATTAGGGAATAACCCGGT
P. gingivalis	ACAGAGGGGGATAACCCGTT
P. asaccharolyticus	TTAGTGGTGAATAACCCGAT
P. bennonis	ACAGAGGAGAATAACCCGGA

f: Reverse primer for *Porphyromonas gingivalis*
PGR : ATGCAATACTCGTATCGCC 476-458bp

P. uenonis	-AACGNCTA-CACGTAGACGA
P. endodontalis	TCTCAGCTA-CACGTAGCTGC
P. gingivalis	ATGCAA-TA-CTCGTATCGCC
P. asaccharolyticus	-AANACCTA-CACGTAGGTGA
P. bennonis	-AACTCCCATCACGTGGAAGT

g: Reverse primer for *Porphyromonas asaccharolyticus*
PAF : TACTCCTTAGATCCCATGAG 183-202bp

P. uenonis	CTCTCCTTAGATCACATGAG
P. endodontalis	TTCTTCTTTCTCCGCATG-G
P. gingivalis	CACTTGTATTATTGCATGAT
P. asaccharolyticus	TACTCCTTAGATCCCATGAG
P. bennonis	CACTCTTAAGTACGCCTGTA

h: Reverse primer for *Porphyromonas asaccharolyticus*
PAR : CTAGAGAGTTTCAAAGGCAAGA 649-628bp

P. uenonis	CTAGAGAGTTTCAAAGGCAAGA
P. endodontalis	CATATCAGTTTCAACGGCAAGA
P. gingivalis	CCCGGCAGTTTCAACGGCAGGC
P. asaccharolyticus	CTAGAGAGTTTCAAAGGCAAGA
P. bennonis	TCTTGCAGTTTCAACGGCAAGG

i: Forward primer for *Porphyromonas bennonis*
PBF : CTTAAGTACGCCTGTACATG 170-189bp

P. uenonis	CCTTAGATCACATGAGAAGA
P. endodontalis	TCTTTCTCCGCATG-GAGGA
P. gingivalis	TGTATTATTGCATGATATTA
P. asaccharolyticus	CCTTAGATCCCATGAGAAGA
P. bennonis	CTTAAGTACGCCTGTACATG

j: Reverse primer for *Porphyromonas bennonis*
PBR : GGTTTCCCAAGAGGCTCAC 1000-982bp

P. uenonis	AGCC-TC-———ATGCAT
P. endodontalis	ATCTGCC-———AT-CAT
P. gingivalis	A-CCATC-———AGTCAT
P. asaccharolyticus	AGCC-TC-———ATGCAT
P. bennonis	GGTTTCCCAAGAGGCTCAC

Fig. 1 Locations and sequences of species-specific primers for the 16S rDNA of *Porphyromonas* species. The nucleotide sequence of each primer has been underlined.

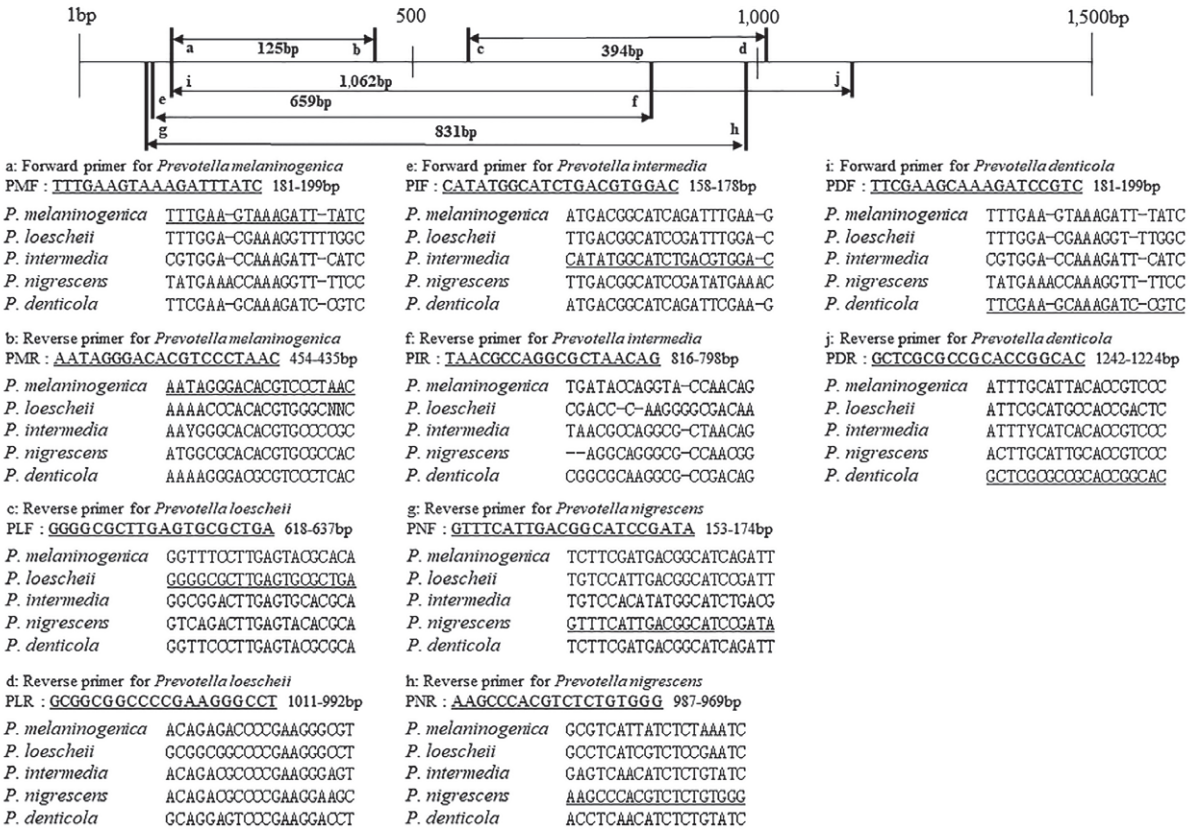


Fig. 2 Locations and sequences of species-specific primers for the 16S rDNA of *Prevotella* species. The nucleotide sequence of each primer has been underlined.

bennonis, whereas the specific reverse primers were designated as PUR for *P. uenonis*, PER for *P. endodontalis*, PGR for *P. gingivalis*, PAR for *P. asaccharolyticus*, and PBR for *P. bennonis*. The amplicon sizes of *P. uenonis*, *P. endodontalis*, *P. gingivalis*, *P. asaccharolyticus*, and *P. bennonis* were 189 bp, 257 bp, 338 bp, 466 bp, and 831 bp, respectively. The specific forward primers of *Prevotella* group were designated as PMF for *P. melaninogenica*, PLF for *P. loescheii*, PIF for *P. intermedia*, PNF for *P. nigrescens*, and PDF for *P. denticola*, whereas the specific reverse primers were designated as PMR for *P. melaninogenica*, PLR for *P. loescheii*, PIR for *P. intermedia*, PNR for *P. nigrescens*, and PDR for *P. denicolla*. The amplicon sizes of *P. melaninogenica*, *P. loescheii*, *P. intermedia*, *P. nigrescens*, and *P. denticola* were 274 bp, 394 bp, 659 bp, 835 bp, and 1,062 bp, respectively.

3.2. Multiplex PCR

3.2.1. Detection limit

Our multiplex PCR method for identifying and detecting ten BPAR successfully amplified DNA fragments of the expected size for each species (Fig. 3, 4). The detection limit was assessed in the presence of titrated bacterial cells, and the sensitivity of the PCR assay was between 5×1 and 5×10 CFU per PCR template (5.0 μl) for the *P. asaccharolyticus*-specific primer set with strain JCM 6326, the *P. endodontalis*-specific primer set with strain ATCC 35406, the *P. loescheii*-specific primer set with strain ATCC 8530, the *P. intermedia*-specific primer set with strain ATCC 25611, the *P. nigrescens*-specific primer set with strain JCM 6322, the *P. gingivalis*-specific primer set with strain ACTT 33277, the *P. uenonis*-specific primer set with strain JCM 13868, the *P. bennonis*-specific primer set with strain JCM 16335, the *P. melaninogenica*-specific primer set with strain JCM 6325, and the *P. denticola*-specific primer set with

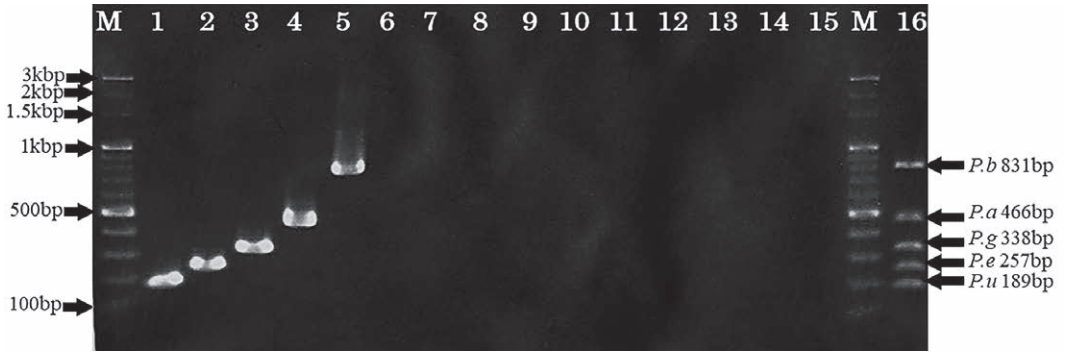


Fig. 3 Multiplex PCR assay for detecting *Phophyromoans* group
The primer mixture contained PUF, PUR, PEF, PER, PGF, PGR, PAF, PAR, PBF, and PBR.
Lanes: 1, *Porphyromonas uenonis* JCM 13868; 2, *Porphyromonas endodontalis* ATCC 35406; 3, *Porphyromonas gingivalis* ATCC 33277; 4, *Porphyromonas asaccharolyticus* JCM 6326; 5, *Porphyromonas bennonis* JCM 16335; 6, *Prevotella melaninogenica* JCM 6325; 7, *Prevotella loescheii* JCM 8530; 8, *Prevotella intermedia* ATCC 25611; 9, *Prevotella nigrescens* JCM 6322; 10, *Prevotella denticola* JCM 13449; 11, *Prevotella oralis* ATCC17929; 12, *Prevotella heparinolytica* ATCC 12102; 13, *Prevotella buccae* JCM 3067; 14, *Prevotella oris* ATCC 14266; 15, *Prevotella veroralis* ATCC 33449; 16, Mixture of *Porphyromonas uenonis* JCM 13868, *Porphyromonas endodontalis* ATCC 35406, *Porphyromonas gingivalis* ATCC 33277, *Porphyromonas asaccharolyticus*, and *Porphyromonas bennonis* JCM 16335. M, molecular size marker（100bp DNA ladder）.

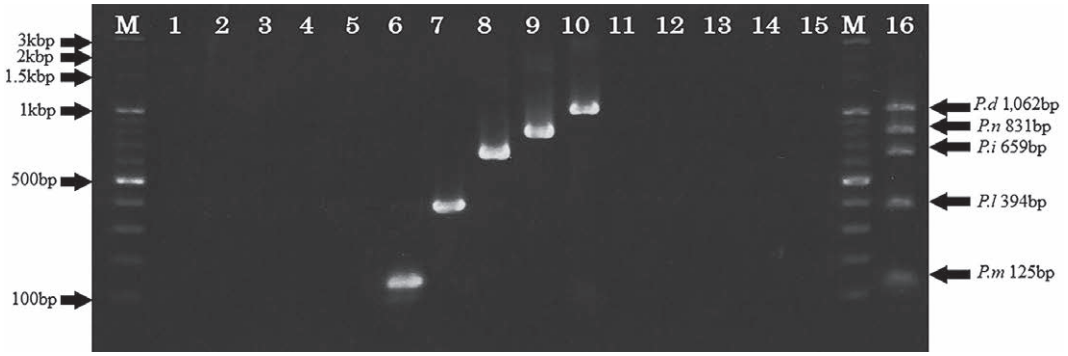


Fig. 4 Multiplex PCR assay for detecting *Prevotella* group
The primer mixture contained PMF, PMR, PLF, PLR, PIF, PIR, PNF, PNR, PDF, and PDR.
Lanes: 1, *Porphyromonas uenonis* JCM 13868; 2, *Porphyromonas endodontalis* ATCC 35406; 3, *Porphyromonas gingivalis* ATCC 33277; 4, *Porphyromonas asaccharolyticus* JCM 6326; 5, *Porphyromonas bennonis* JCM 16335; 6, *Prevotella melaninogenica* JCM 6325; 7, *Prevotella loescheii* JCM 8530; 8, *Prevotella intermedia* ATCC 25611; 9, *Prevotella nigrescens* JCM 6322; 10, *Prevotella denticola* JCM 13449; 11, *Prevotella oralis* ATCC17929; 12, *Prevotella heparinolytica* ATCC 12102; 13, *Prevotella buccae* JCM 3067; 14, *Prevotella oris* ATCC 14266; 15, *Prevotella veroralis* ATCC 33449; 16, Mixture of *Prevotella melaninogenica* JCM 6325, *Prevotella loescheii* JCM 8530, *Prevotella intermedia* JCM 6322, *Prevotella nigrescens*, and *Prevotella denticola* JCM 13449. M, molecular size marker（100bp DNA ladder）.

strain JCM 13449（Fig. 5, 6）.

3.2.2. Assay of representative Prevotella species other than BPAR

As representative *Prevotella* species other than BPAR, *Prevotella oralis*, *Prevotella heparinolytica*, *Prevotella buccae*, *Prevotella oris*, and *Prevotella veroralis* were subjected to PCR using the designed primer sets. However, no amplicons were produced from any of representative *Prevotella* species other than BPAR（Fig. 3, 4）.

4. Discussion

BPAR including *Porphyromonas* and some *Prevotella* species have been implicated in endodontic infections associated with pain and purulent abscesses[10–12]. This group of microorganisms includes *P. endodontalis*, *P. gingivalis*, *P. intermedia*, and *P. nigrescens*. Moreover, some BPAR are major pathogens in destructive peri-

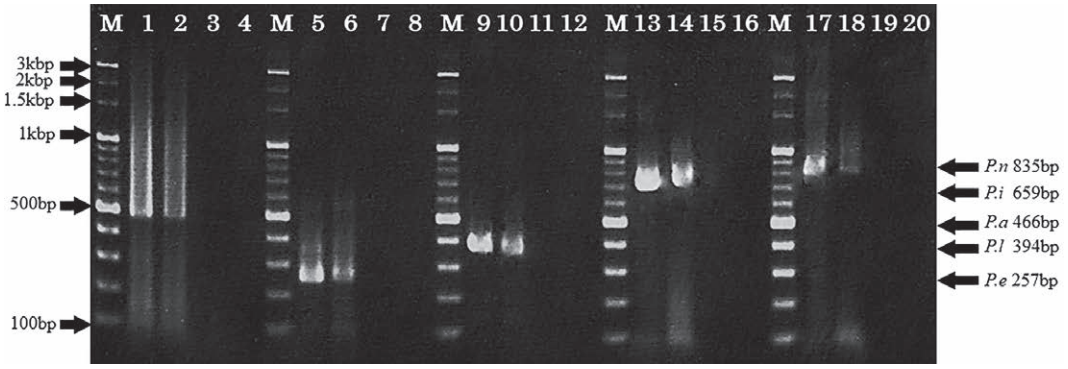


Fig. 5 Detection limit of the multiplex PCR assay for detecting *P. asaccharolytica, P. endodontalis, P. loescheii, P. intermedia, P. nigrescens*

The primer mixture contained PUF, PUR, PEF, PER, PGF, PGR, PAF, PAR, PBF, and PBR for *Porphyromonas* species, and PMF, PMR, PLF, PLR, PIF, PIR, PNF, PNR, PDF, and PDR for *Prevotella* species.

Lanes 1–4, *Porphyromonas asaccharolyticus* JCM 6326; Lanes 5–8, *Porphyromonas endodontalis* ATCC 35406; Lanes 9–12 *Prevotella loescheii* JCM 8530; Lanes 13–16 *Prevotella intermedia* ATCC 25611; Lanes 17–20 *Prevotella nigrescens* JCM 6322. The following numbers of cells were added: 5×10^2 (lanes 1, 5, 9, 13, 17), 5×10 (lanes 2, 6, 10, 14, 18), 5×1 (lanes 3, 7, 11, 15, 19), 0 (lanes 4, 8, 12, 16, 20). M, molecular size marker (100 bp DNA ladder).

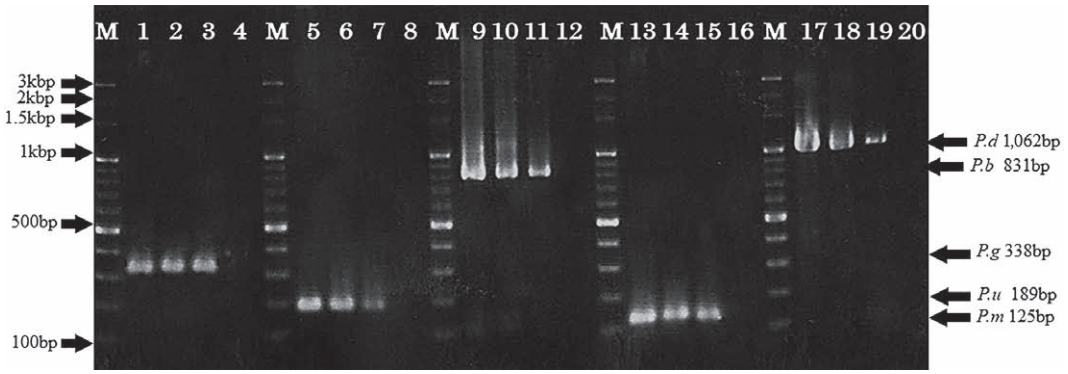


Fig. 6 Detection limit of the multiplex PCR assay for detecting *P. gingivalis, P. uenonis, P. bennonis, P. melaninogenica, P. denticola*

The primer mixture contained PUF, PUR, PEF, PER, PGF, PGR, PAF, PAR, PBF, and PBR for *Porphyromonas* species, and PMF, PMR, PLF, PLR, PIF, PIR, PNF, PNR, PDF, and PDR for *Prevotella* species.

Lanes 1–4, *Porphyromonas gingivalis* ATCC 33277; Lanes 5–8, *Porphyromonas uenonis* JCM 13868; Lanes 9–12 *Porphyromonas bennonis* JCM 16335; Lanes 13–16, *Prevotella melaninogenica* JCM 6325; Lanes 17–20 *Prevotella denticola* JCM 13449. The following numbers of cells were added: 5×10^2 (lanes 1, 5, 9, 13, 17), 5×10 (lanes 2, 6, 10, 14, 18), 5×1 (lanes 3, 7, 11, 15, 19), 0 (lanes 4, 8, 12, 16, 20). M, molecular size marker (100 bp DNA ladder).

odontal disease[6, 13] and have been recovered from infections of the respiratory tract, the female genital tract and the intestinal tract[14]. The organisms display a wide variety of virulence factors that may be pertinent to acute endodontic infections[15]. *P. asaccharolyticus* is rarely found in the human oral microbiota and that it is not able to colonize periodontal pockets[6, 16, 17]. *P. loescheii* and *P. denticola* have been isolated from healthy sites albeit in smaller proportions[18]. *P. melaninogenica* is commonly found in the oral cavity of healthy individuals and is the most frequently identified black-pigmented species in the dental plaque of children between 7 and 9 years of age[19–21]. *P. bennonis* were isolated in mixed culture from various skin and soft-tissue infections of non-oral origin, mainly from abscesses in the buttock and groin areas[22]. *P. uenosis* were found as part of a mixed flora in various infections, which apparently have their origin in the intestinal tract[23]. It remains unknown whether *P. bennonis* and *P. uenosis* are part of normal oral flora. Moreover, the distribution of BPAR in different oral sites

and their role in common oral afflictions, particularly periodontal diseases, remain equivocal.

A PCR method has been applied for the rapid and specific detection of periodontopathic bacteria in subgingival plaque and is potentially of clinical benefit in the diagnosis and treatment of periodontitis subjects. However, several technical points need to be modified before the conventional PCR detection system can be used by clinicians. To develop a PCR-based technique more applicable for clinical use than conventional PCR, we established a multiplex PCR for the representative BPAR, i.e. five *Prevotella* species and five *Porphyromonas* species, using only two PCR tubes per sample. A multiplex-PCR method is a rapid tool that allows for the simultaneous amplification of more than one sequence of target DNA in a single reaction, thereby saving time and reagents[24].

In the present study, we designed species-specific primers with the already mentioned means, for the identification and detection of ten BPAR using a PCR method. These primers were able to distinguish each BPAR and did not display cross-reactivity with representative *Prevotella* species other than BPAR. Moreover, we developed a multiplex PCR method with the ability to identify and differentiate *Porphyromonas* and *Prevotella* species (i.e. *P. uenonis*, *P. endodontalis*, *P. gingivalis*, *P. asaccharolyticus*, *P. bennonis*, *P. melaninogenica*, *P. loescheii*, *P. intermedia*, *P. nigrescens*, and *P. denticola*) using only each one PCR tubes per sample.

Our multiplex PCR method is easy because the use of MightyAmp DNA Polymerase Ver.3 (Takara) means that DNA extraction may be avoided, and species identification and detection using this method only takes approximately 2 hours. Thus, the method described herein will allow the prevalence of the representative BPAR and their involvement in oral infections to be fully clarified in future studies.

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