*Oral Microbiology Immunology 2005: 20: 43–46*
*Printed in Singapore. All rights reserved*



**Oral Microbiology and Immunology**

Short communication

# Multiplex polymerase chain reaction assay for simultaneous detection of black-pigmented *Prevotella* species in oral specimens

**A. Yoshida, M. Tachibana, T. Ansai, T. Takehara**
Department of Preventive Dentistry, Kyushu Dental College, Kitakyushu, Japan

*Yoshida A, Tachibana M, Ansai T, Takehara T. Multiplex polymerase chain reaction assay for simultaneous detection of black-pigmented* Prevotella *species in oral specimens. Oral Microbiol Immunol 2005: 20: 43–46.* © Blackwell Munksgaard, 2005.

*Prevotella* species are a major component of the oral microflora and some have been implicated in various forms of periodontal disease. Despite the importance of understanding the prevalence of these organisms in the oral microflora, no rapid, simultaneous detection system for these species has been reported. This study developed a multiplex polymerase chain reaction assay for the simultaneous detection of four oral black-pigmented *Prevotella* species in various oral specimens. This assay will be useful for determining the prevalence of these organisms in the oral ecosystem. Furthermore, this assay system should prove a useful tool for analyzing the role of black-pigmented *Prevotella* species in the mouth.

Key words: multiplex polymerase chain reaction assay; *Prevotella intermedia*; *Prevotella loescheii*; *Prevotella melaninogenica*; *Prevotella nigrescens*

A. Yoshida, Department of Preventive Dentistry, Kyushu Dental College, 2-6-1 Manazuru, Kokurakita-ku, Kitakyushu 803-8580, Japan
Tel.: +81 93 5821131 (Ext. 2103);
fax: +81 93 5917736;
e-mail: akihiro@kyu-dent.ac.jp
Accepted for publication August 5, 2004

*Prevotella* species form a major portion of the microflora of the human oral cavity (3, 8, 17, 25). Of these, *Prevotella intermedia* and *Prevotella nigrescens*, black-pigmented saccharolytic anaerobic rods that have been recognized previously as two genotypes of *P. intermedia* (genotype I corresponds to *P. intermedia* and genotype II to *P. nigrescens*) (9, 20), seem to play an important etiologic role in periodontitis (22, 27). There are an increasing number of reports that *P. intermedia* is associated with periodontitis, whereas *P. nigrescens* is a natural commensal of the gingival sulcus and supragingival plaque (7, 10, 12), although there is also evidence countering this theory (24). Therefore, these species are either opportunistic pathogens or they are heterogeneous species with respect to virulence (6).

Methods for detecting bacteria include direct microscopy, culture, enzymatic activity, enzyme-linked immunosorbent assay, and DNA probes (11, 13, 16, 29). The polymerase chain reaction (PCR) is one of the most sensitive and rapid methods for determining the prevalence of periodontal bacteria (4, 26).

To clarify the etiologic role of *Prevotella* species in periodontal sites, an analysis of the symbiotic relationships among these species is required. Therefore, we developed a multiplex PCR assay system for the simultaneous detection of four black-pigmented *Prevotella* species. This is the first investigation of the simultaneous detection of several *Prevotella* species using multiplex PCR.

The bacterial strains studied are listed in Table 1. The *P. intermedia*, *P. nigrescens*, *Prevotella melaninogenica*, and *Prevotella loescheii* strains were cultured anaerobically (10% $CO_2$, 10% $H_2$, 80% $N_2$) at 37°C in GAM broth (Nissui Medical Co., Tokyo, Japan) supplemented with hemin (5 μg/ml) and menadione (0.5 μg/ml). Genomic DNA was isolated and purified using an IsoQuick Nucleic Acid Extraction Kit (ORCA Research Inc., Bothell, WA), in accordance with the manufacturer's instructions. Human saliva, tongue coat, and subgingival plaque were prepared as follows. Five hundred microliters of saliva and the same amount of phosphate-buffered

*Table 1.* Bacterial strains and specificity of the primers

| Strain | Source or distribution | Amplification with primer* | | | |
|---|---|---|---|---|---|
| | | *P. intermedia* | *P. nigrescens* | *P. melaninogenica* | *P. loescheii* |
| *P. intermedia* ATCC 25611 | KDC[a] | + | – | – | – |
| *P. nigrescens* ATCC 25261 | ATCC[b] | – | + | – | – |
| *P. melaninogenica* ATCC 25845 | ATCC | – | – | + | – |
| *P. loescheii* ATCC 15930 | ATCC | – | – | – | + |

*Strains not showing amplification (source or distribution in parentheses): *Prevotella denticola* ATCC 33185 (ATCC), *Prevotella corporis* ATCC 33547 (ATCC), *Prevotella pallens* ATCC 700821 (ATCC), *Prevotella oris* ATCC 33573 (ATCC), *Prevotella veroralis* ATCC 33779 (ATCC), *Prevotella oralis* ATCC 33322 (ATCC), *Prevotella bivia* ATCC 29303 (ATCC), *Porphyromonas gingivalis* W83 (KU)[c], *Porphyromonas gingivalis* W50 (KU), *Porphyromonas gingivalis* 381 (KU), *Porphyromonas gingivalis* ATCC 33277 (KDC), *Porphyromonas gingivalis* ATCC 49417 (KU), *Tannerella forsythia* ATCC 43037 (KU), *Actinobacillus actinomycetemcomitans* Y4 (KU), *Fusobacterium nucleatum* ATCC 10953 (KU), *Treponema denticola* ATCC 35404 (KU), *Treponema denticola* ATCC 35405 (KU), *Actinomyces naeslundii* ATCC 12104 (KU), *Actinomyces naeslundii* ATCC 15988 (KU), *Eikenella corrodens* 1085 (KU), *Streptococcus mutans* GS-5 (KU), *Streptococcus sobrinus* 6715 (KU), *Escherichia coli* DH5$^{\alpha}$ (Invitrogen).

[a]KDC, culture collection in the Department of Preventive Dentistry, Kyushu Dental College, Kitakyushu, Japan.

[b]ATCC, American Type Culture Collection, Manassas, VA.

[c]KU, culture collection in the Department of Preventive Dentistry, Kyushu University Faculty of Dental Science, Fukuoka, Japan.

*Table 2.* Oligonucleotide primers

| Oligonucleotide primers[a] | Sequence | Amplicon size (bp) | Gene[b] | Reference |
|---|---|---|---|---|
| *Pi phoC* 51F | 5′-GAC TTT TGC ACA GAA TGC A-3′ | 718 | *phoC* | 3, 5 |
| *Pi phoC* 768R | 5′-CTT GGC AAC CTT GCC TTC-3′ | | | |
| *Pn* 16S 188F | 5′-TTT CAT TGA CGG CAT CCG-3′ | 825 | 16S rRNA | 18, 21 |
| *Pn* 16S 1012R | 5′-CAC GTC TCT GTG GGC AG-3′ | | | |
| *Pm phyA* 132F | 5′-CGT CAT GAA GGA GAT TGG-3′ | 389 | *phyA* | 1 |
| *Pm phyA* 520R | 5′-ATA GAA CCG TCA ACG CTC-3′ | | | |
| *Pl plaA* 186F | 5′-TGC CAA CTC CCG ATT TC-3′ | 637 | *plaA* | 15 |
| *Pl plaA* 822R | 5′-TAC ACC AAG GTT TTC CCC-3′ | | | |

[a] Abbreviations: *Pi, P. intermedia, Pn, P. nigrescens; Pm, P. melaninogenica; Pl, P. loescheii.*

[b]Accession numbers are AB017537 for *P. intermedia phoC* gene, L16471 for *P. nigrescens* 16S rRNA gene, U27587 for *P. melaninogenica phyA* gene, and AF080443 for *P. loescheii plaA* gene.

saline (PBS; 0.12 M NaCl, 0.01 M $Na_2H$-$PO_4$, 5 mM $KH_2PO_4$ [pH 7.5]) were mixed and centrifuged at 12,000 × *g* for 10 min. To the precipitate, 500 µl of cell lysis buffer (1.0% Triton X-100, 20 mM Tris-HCl, 2 mM EDTA [pH 8.0]) was added. The precipitate was vortexed and the chromosomal DNA from the bacteria was extracted by boiling at 100°C for 10 min. Tongue coat samples were collected from the tongue dorsum. Saliva was removed from the tongue coat using air, and 1 mg (wet weight) of tongue coat was washed with PBS twice. The precipitate was suspended in 100 µl of cell lysis solution. The lysate was boiled at 100°C for 10 min and the chromosomal DNA was extracted. Subgingival plaque samples were obtained by inserting a sterile endodontic paperpoint into the subgingival site for 10 s. The paperpoint was transferred into 200 µl of PBS and centrifuged at 12,000 × *g* for 5 min. The cells were resuspended in 100 µl of cell lysis buffer, boiled at 100°C for 5 min, and the supernatant used as the PCR template (23, 28).

The oligonucleotide primers and their DNA sequences are listed in Table 2. The *P. intermedia*-, *P. nigrescens*-, *P. melaninogenica*-, and *P. loescheii*-specific primer-pairs were designed from the *phoC* (5), 16S rRNA (18, 21), *phyA* (1), and *plaA* (15) genes, respectively. The genes *phoC*, *phyA*, and *plaA* encode *P. intermedia* acid phosphatase, *P. melaninogenica* hemolysin, and *P. loescheii* adhesin precursor, respectively. The specificities of the primers were initially confirmed using BLAST (2) at the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/). They were then confirmed using conventional PCR (Table 1) as follows: 95°C for 5 min, followed by 25 cycles of 94°C for 15 s, 54°C for 30 s, and 72°C for 1 min. To confirm the specificity of the PCR products, the DNA was sequenced (data not shown). Furthermore, the primer specificities were confirmed by PCR using chromosomal DNA from various bacteria (Table 1). When the black-pigmented *Prevotella* species-specific primers were used, fragments of the expected size were amplified from *Prevotella* species, but not from other bacteria. These results demonstrated that each set of primers was specific for the black-pigmented *Prevotella* species.

To optimize the multiplex PCR assay, *P. intermedia*, *P. nigrescens*, *P. melaninogenica*, and *P. loescheii* were used. The reaction mixture was made in accordance with the manufacturer's instructions (QIAGEN Multiplex PCR Handbook; Qiagen GmbH, Hilden, Germany). Annealing temperatures of 48–57°C were tested, and the clearest bands were obtained at 54°C. The PCR cycle numbers of 25, 30, and 35 cycles were tested, and all the resultant bands were sufficiently clear after 35 cycles. Finally, multiplex PCR was performed as follows: 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 54°C for 90 s, and 72°C for 90 s. A 50-µl PCR mixture for multiplex PCR consisted of 25 µl 2 × QIAGEN Multiplex PCR Master Mix (Qiagen), 2 µM of each primer, and 1 µl of template DNA. In a 50-µl reaction mixture, 1–10 ng of genomic DNA was used as a template.

Under these conditions, chromosomal DNA of *P. intermedia* ATCC 25611, *P. nigrescens* ATCC 25261, *P. melaninogenica* ATCC 25845, and *P. loescheii* ATCC 15930 were used for multiplex PCR. The resulting amplification products produced single bands at 718, 825, 389, and 637 bp, respectively (Fig. 1), which corresponded to the size of the bands amplified when genomic DNA samples were used.

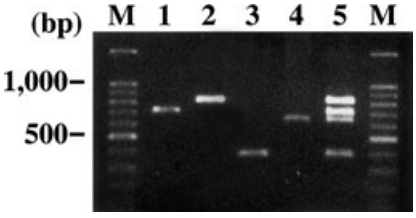


*Fig. 1.* Single and multiplex PCR amplification products for *Prevotella* genes. Lane 1: *P. intermedia* ATCC 25611. Lane 2: *P. nigrescens* ATCC 25261. Lane 3: *P. melaninogenica* ATCC 25845. Lane 4: *P. loescheii* ATCC 15930. Lane 5: multiplex. M, molecular size standard. The DNA fragments were visualized on 0.8% agarose gels stained with ethidium bromide.

Representative detection patterns for *Prevotella* species, using the multiplex PCR assay for clinical specimens from patients with periodontitis, are shown in Fig. 2. The presence of PCR inhibitors in dental plaque was assessed using chromosomal DNA of the four *Prevotella* species. Chromosomal DNA from the four black-pigmented *Prevotella* species spiked with approximately 10 μg (wet weight) of *Prevotella*-negative dental plaque showed no inhibition (data not shown). In addition, the detection patterns of *Prevotella* species in various oral specimens using this assay are shown in Table 3. These results were confirmed by conventional PCR, using each primer pair, and all the results matched the results of the multiplex PCR assay (data not shown). Therefore, this multiplex PCR assay can be used to confirm the presence of these *Prevotella* species in oral specimens. Of the five subgingival sites positive for *P. intermedia*, four were positive for *Porphyromonas gingivalis*. Of the eight subgingival sites positive for *P. nigrescens*, six were positive for *Fusobacterium nucleatum*. Of the eight subgingival sites positive for *P. melaninogenica*, six were positive for *F. nucleatum.* We did not find a clear relationship between black-pigmented *Prevotella* species and *Tannerella forsythia* (formerly *Bacteroides forsythus* or *Tannerella forsythensis*) (14) or *Treponema denticola* (data not shown). This may have been owing to the small number of samples. Further research is required for a detailed analysis of these relationships. A previous study revealed the prevalence of black-pigmented *Prevotella* species in subgingival plaques and fecal samples (19). Further longitudinal studies examining the prevalence and identity of various black-pigmented *Prevotella* species within family members are required.

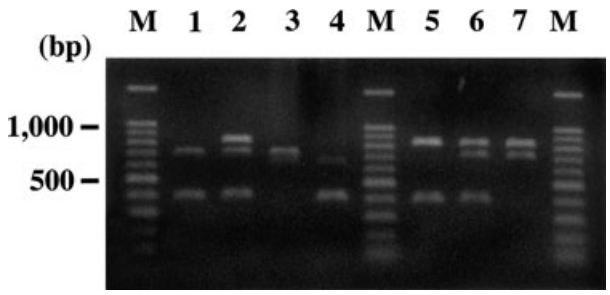


*Fig. 2.* Examples of multiplex PCR amplifications using oral specimens as template. Lanes 1–4: tongue coat (patients Nos. 2, 3, 9, and 10, respectively). Lanes 5–7: subgingival plaque (patients Nos. 1, 2, and 6, respectively). M, molecular size standard. DNA fragments were visualized on 0.8% agarose gels stained with ethidium bromide.

*Table 3.* Simultaneous detection of four *Prevotella* species by multiplex PCR from oral specimens

| Specimen type and patient No. | Amplification with primer | | | |
|---|---|---|---|---|
| | *P. intermedia* | *P. nigrescens* | *P. melaninogenica* | *P. loescheii* |
| Saliva | | | | |
| 1 | – | – | + | – |
| 2 | – | – | – | – |
| 3 | – | – | + | – |
| 4 | – | – | – | – |
| 5 | – | – | + | – |
| 6 | – | – | – | – |
| 7 | – | – | + | – |
| 8 | – | – | – | – |
| 9 | – | – | – | – |
| 10 | – | – | + | – |
| Tongue coat | | | | |
| 1 | + | – | + | – |
| 2 | + | – | + | – |
| 3 | + | + | + | – |
| 4 | – | – | + | – |
| 5 | + | + | + | – |
| 6 | – | – | – | – |
| 7 | – | – | + | – |
| 8 | – | – | + | – |
| 9 | + | – | – | + |
| 10 | – | – | + | + |
| Subgingival plaque | | | | |
| 1 | – | + | + | – |
| 2 | + | + | + | – |
| 3 | – | + | + | – |
| 4 | + | + | + | – |
| 5 | – | + | + | – |
| 6 | + | + | – | – |
| 7 | – | + | + | – |
| 8 | + | + | + | – |
| 9 | + | – | – | – |
| 10 | – | – | + | – |

Our results indicate that the multiplex PCR assay is a sensitive, specific, and highly effective diagnostic tool for the simultaneous identification of oral black-pigmented *Prevotella* species. The role of the black-pigmented *Prevotella* species in periodontitis is still unclear. This simultaneous detection assay will contribute to the elucidation of the symbiotic relationships of *Prevotella* species in periodontal sites and other oral flora.

## Acknowledgments

We thank Dr. Okamoto, Department of Oral Microbiology, Tsurumi University, for useful suggestions regarding this research. This investigation was supported by Grants-in-Aid (B) 14370700 (to T. Takehara) and (C) 13672164 (to T. Ansai) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, by a research grant from the Nakatomi Foundation (to A. Yoshida), and by a research grant from the Clinical Research Foundation (to A. Yoshida).

## References

1. Allison HE, Hillman JD. Cloning and characterization of a *Prevotella melaninogenica* hemolysin. Infect Immun 1997: **65**: 2765–2771.
2. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997: **25**: 3389–3402.
3. Ansai T, Awano S, Chen X, Fuchi T, Arimoto T, Akifusa S, et al. Purification and characterization of alkaline phosphatase containing phosphotyrosyl phosphatase activity from the bacterium *Prevotella intermedia*. FEBS Lett 1998: **428**: 157–160.

4. Ashimoto A, Chen C, Bakker I, Slots J. Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions. Oral Microbiol Immunol 1996: **11**: 266–273.
5. Chen X, Ansai T, Awano S, Iida T, Barik S, Takehara T. Isolation, cloning, and expression of an acid phosphatase containing phosphotyrosyl phosphatase activity from *Prevotella intermedia*. J Bacteriol 1999: **181**: 7107–7114.
6. Dahlen GG, Johnson JR, Gmür R. *Prevotella intermedia* and *Prevotella nigrescens* serotypes, ribotypes and binding characteristics. FEMS Microbiol Lett 1996: **138**: 89–95.
7. Dahlen G, Wikstrom M, Renvert S, Gmür R, Guggenheim B. Biochemical and serological characterization of *Bacteroides intermedius* strains isolated from the deep periodontal pocket. J Clin Microbiol 1990: **28**: 2269–2274.
8. Finegold SM, Strong CA, McTeague M, Marina M. The importance of black-pigmented gram-negative anaerobes in human infections. FEMS Immunol Med Microbiol 1993: **6**: 77–82.
9. Frandsen EV, Poulsen K, Kilian M. Confirmation of the species *Prevotella intermedia* and *Prevotella nigrescens*. Int J Syst Bacteriol 1995: **45**: 429–435.
10. Gmür R, Guggenheim B. Interdental supragingival plaque – a natural habitat of *Actinobacillus actinomycetemcomitans, Bacteroides forsythus, Campylobacter rectus,* and *Prevotella nigrescens*. J Dent Res 1994: **73**: 1421–1428.
11. Guillot E, Mouton C. PCR–DNA probe assays for identification and detection of *Prevotella intermedia sensu stricto* and *Prevotella nigrescens*. J Clin Microbiol 1997: **35**: 1876–1882.
12. Hanazawa S, Tanaka S, Kin M, Amano S, Nakada K, Masuda T, et al. Application of monoclonal antibodies to the detection of black-pigmented *Bacteroides* spp. in subgingival plaques by immunoslot blot assay. J Clin Microbiol 1990: **28**: 2248–2252.
13. Lamster IB, Oshrain RL, Harper DS, Celenti RS, Hovliaras CA, Gordon JM. Enzyme activity in crevicular fluid for detection and prediction of clinical attachment loss in patients with chronic adult periodontitis. Six month results. J Periodontol 1988: **59**: 516–523.
14. Maiden MF, Cohee P, Tanner AC. Proposal to conserve the adjectival form of the specific epithet in the reclassification of *Bacteroides forsythus* Tanner et al. 1986 to the genus *Tannerella* Sakamoto et al. 2002 as *Tannerella forsythia* corrig., gen. nov., comb. nov. Request for an Opinion. Int J Syst Evol Microbiol 2003: **53**: 2111–2112.
15. Manch-Citron JN, Allen J, Moos M Jr, London J. The gene encoding a *Prevotella loescheii* lectin-like adhesin contains an interrupted sequence which causes a frameshift. J Bacteriol 1992: **174**: 7328–7336.
16. Melvin WL, Assad DA, Miller GA, Gher ME, Simonson L, York AK. Comparison of DNA probe and ELISA microbial analysis methods and their association with adult periodontitis. J Periodontol 1994: **65**: 576–582.
17. Nakagawa S, Fujii H, Machida Y, Okuda K. A longitudinal study from prepuberty to puberty of gingivitis. Correlation between the occurrence of *Prevotella intermedia* and sex hormones. J Clin Periodontol 1994: **21**: 658–665.
18. Okamoto M, Maeda N, Kondo K, Leung KP. Hemolytic and hemagglutinating activities of *Prevotella intermedia* and *Prevotella nigrescens*. FEMS Microbiol Lett 1999: **178**: 299–304.
19. Okuda K, Fukumoto Y, Takazoe I. Enumeration of cultivable black-pigmented *Bacteroides* species in human subgingival dental plaque and fecal samples. Oral Microbiol Immunol 1988: **3**: 28–31.
20. Shah HN, Gharbia SE. Biochemical and chemical studies on strains designated *Prevotella intermedia* and proposal of a new pigmented species, *Prevotella nigrescens* sp. nov. Int J Syst Bacteriol 1992: **42**: 542–546.
21. Shah HN, Gharbia SE, Scully C, Finegold SM. Oligonucleotide probes to the 16S ribosomal RNA. implications of sequence homology and secondary structure with particular reference to the oral species *Prevotella intermedia* and *Prevotella nigrescens*. Oral Dis 1995: **1**: 32–36.
22. Slots J, Bragd L, Wikstrom M, Dahlen G. The occurrence of *Actinobacillus actinomycetemcomitans*, *Bacteroides gingivalis* and *Bacteroides intermedius* in destructive periodontal disease in adults. J Clin Periodontol 1986: **13**: 570–577.
23. Suzuki N, Nakano Y, Yoshida Y, Ikeda D, Koga T. Identification of *Actinobacillus actinomycetemcomitans* serotypes by multiplex PCR. J Clin Microbiol 2001: **39**: 2002–2005.
24. Teanpaisan R, Douglas CW, Walsh TF. Characterisation of black-pigmented anaerobes isolated from diseased and healthy periodontal sites. J Periodontal Res 1995: **30**: 245–251.
25. van Winkelhoff AJ, van Steenbergen TJ, de Graaff J. The role of black-pigmented *Bacteroides* in human oral infections. J Clin Periodontol 1988: **15**: 145–155.
26. Watanabe K, Frommel TO. Detection of *Porphyromonas gingivalis* in oral plaque samples by use of the polymerase chain reaction. J Dent Res 1993: **72**: 1040–1044.
27. Wennstrom JL, Dahlen G, Svensson J, Nyman S. *Actinobacillus actinomycetemcomitans*, *Bacteroides gingivalis* and *Bacteroides intermedius*: predictors of attachment loss? Oral Microbiol Immunol 1987: **2**: 158–162.
28. Yoshida A, Suzuki N, Nakano Y, Oho T, Kawada M, Koga T. Development of a 5′ fluorogenic nuclease-based real-time PCR assay for quantitative detection of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*. J Clin Microbiol 2003: **41**: 863–866.
29. Zambon JJ. Principles of evaluation of the diagnostic value of subgingival bacteria. Ann Periodontol 1997: **2**: 138–148.