Simultaneous Detection of *Bacteroides forsythus* and *Prevotella intermedia* by 16S rRNA Gene-Directed Multiplex PCR

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Received 31 July 1998/Returned for modification 2 November 1998/Accepted 13 February 1999

In a 16S rRNA gene-directed multiplex PCR, *Prevotella intermedia-* and *Bacteroides forsythus*-specific reverse primers were combined with a single conserved forward primer. A 660-bp fragment and an 840-bp fragment that were specific for both species could be amplified simultaneously. A total of 152 clinical samples, subgingival plaque and swabs of three different oral mucosae, from 38 periodontitis patients were used for the evaluation.

The major putative pathogens known to be involved in destructive periodontal diseases include *Actinobacillus actinomycetemcomitans, Bacteroides forsythus, Campylobacter rectus, Eikenella corrodens, Fusobacterium nucleatum, Porphyromonas gingivalis, Prevotella intermedia,* and spirochetes (8). Various methods for the detection of these pathogens have been described (30), but there is no consensus regarding the method of choice. Approaches frequently used for microbiological studies include direct microscopy, cultivation, enzyme tests, enzyme-linked immunosorbent assay, detection of signature sequences by using genomic or oligonucleotide probes, and the PCR. The latter, because of its sensitivity of as few as 3 to 50 CFU, is of particular interest for studying the early colonization of the host with periodontal pathogens or suppression of the pathogens following periodontal treatment. Most PCR studies have concentrated on the detection of a single pathogenic species using various targets for primer annealing: *A. actinomycetemcomitans* (leukotoxin gene-directed primers [11, 12] and 16/23S rRNA gene [rDNA]-directed primers [19]), *B. forsythus* (randomly amplified polymorphic DNA [RAPD] marker flanking primers [5, 15] and 16S rDNA [23]), *P. gingivalis* (RAPD marker flanking primers [3], collagenase gene-directed primers [4, 29], outer membrane protein gene-directed primers [17], and 16/23S rDNA-directed primers [21, 22, 26]), and *P. intermedia* (RAPD marker flanking primers [16]).

To assess the epidemiology of periodontal pathogens and the diagnosis and treatment of periodontal diseases, most species of etiologic importance need to be detected. In a number of studies, seven or eight putative periodontal pathogens have been detected by individual PCRs (2, 6, 24). To minimize the time and expenditure needed for detection procedures, sets of 16S rDNA-directed primers have been combined to detect more than one species in a single sample. However, this multiplex PCR variant was only evaluated for the species *A. actinomycetemcomitans, E. corrodens,* and *P. gingivalis* (13, 25, 28).

The aim of the present study was to develop a multiplex PCR using one 16S rDNA-directed conserved forward primer combined with species-specific reverse primers for simultaneous detection of *B. forsythus* and *P. intermedia*. After evaluating the method with 6 *B. forsythus* and 10 *P. intermedia* strains, as well as 23 strains of other closely and more distantly related (oral) bacteria, the PCR was applied to a total of 152 clinical samples consisting of 38 samples each of subgingival plaque and swabs of tonsil, cheek, and tongue mucosae.

**Bacterial strains and patient specimens.** To evaluate the multiplex PCR, the bacteria used as positive controls were *B. forsythus* ATCC 43037T, FR001/12-3, FR002/23-2, FR004/13-4, FR007/24-6, and FR009/11-6 and *P. intermedia* ATCC 25611T, A735, FR032/26, FR028/11, H91-360/1, H91-1880/2, Hg404, Hg1103, Hg1269, and MH6. The bacteria used as negative controls were *A. actinomycetemcomitans* ATCC 33384T, *Actinomyces israelii* ATCC 12102T, *A. gerencseriae* ATCC 23860T, *A. odontolyticus* DSM43331, *Capnocytophaga gingivalis* ATCC 33624T, *C. granulosa* ATCC 51502T, *C. haemolytica* ATCC 51501T, *C. ochracea* ATCC 33596, *C. putigena* ATCC 33612T, *E. corrodens* ATCC 23834T, *F. nucleatum* ATCC 25586T, *Hae-mophilus aphrophilus* ATCC 33894T, *P. intermedia* GH399, *Stomatococcus mucllaginosus* MCCM00557, *Streptococcus intermedius* DSM20573, *S. mutans* NCTC11060, and *S. salivarius* DSM20068. Strains were obtained from the following sources: ATCC strains, American Type Culture Collection, Manassas, Va.; NCTC strains, National Collection of Type Cultures, London, United Kingdom; DSM strains, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; FR strains, K. Pelz, Institute of Medical Microbiology and Hygiene, Freiburg, Germany; A strains, I. Mitchelmore, St. Bartholomew’s Hospital Medical College, London, United Kingdom; the GH strain, G. Haase, Institute of Medical Microbiology, Aachen, Germany; H strains, C. Hoehne, Institute of Medical Microbiology, Halle, Germany; Hg strains, J. T. M. Van Steenbergen, Academic Center for Dentistry, Amsterdam, The Netherlands; MCMC strains, Medical Culture Collection Marburg, Marburg, Germany; MH strains, M. Haapasaylo, Department of Cariology, University of Helsinki, Helsinki, Finland.

All bacteria were grown on Columbia agar with 5% sheep blood for 3 to 5 days at 37°C. After sufficient growth, 1 to 10 colonies (depending on their size) of the pure bacterial cul-
tures were suspended in 250 μl of reduced transport medium (20) and the suspensions were kept frozen (−70°C) until investi-
gation.

A total of 152 clinical samples were taken from 38 adult patients with untreated periodontitis, representing consecutive
samples recruited from the Department of Periodontology, Julius Maximalian University, Würzburg, Germany. The mean
age of the patients was 51.8 ± 11.0 years, and 21 were female
and 17 were male. The mean percentage of sites with a peri-
odontal probing depth of 4 to 6 mm was 31.4 ± 11.9, the mean
percentage of sites with a periodontal probing depth of ≥7 mm
was 4.5 ± 6.3, and the mean percentage of sites with bleeding
on probing was 53.8 ± 32.3. Patients who had used systemic
antibiotics in the previous 6 months were excluded from the
study. All patients enrolled in the study signed the informed
consent form approved by the Ethics Committee of the Med-
ical Faculty, Julius Maximalian University. Subgingival plaque
samples were obtained with a sterile curette from the four
deepest periodontal pockets, pooled, and placed in 1 ml of
reduced transport medium. Samples from oral mucous mem-
brane, were collected with sterile cotton swabs, and
samples were obtained with a sterile curette from the four
sites of primers were determined for both test species. The
specificity and sensitivity of the PCR. The specificity of the
PCR was evaluated by testing 6 B. forsythus and 10 P. interme-
dia strains, as well as 23 representatives of closely or more
distantly related species (aliquots of 100 ng of nucleic acid).
Amplicons appearing to be of the expected sizes (P. intermedia,
660 bp; B. forsythus, 840 bp) were found with all of the strains
tested. Neither of these two PCR products or other PCR bands
were pelleted by centrifugation for 10 min at 5,000 × g, and
were separated electrophoretically on a 2% agarose gel
at 72°C was added. Amplification products (aliquots of 10 μl)
were separated electrophoretically on a 2% agarose gel
and after a 5-day incubation for 37°C. The original cul-
tures of both microorganisms and the dilutions were mixed
(1:1) by vortexing before samples were taken for DNA extrac-
tion and the subsequent multiplex PCR. The colonies on Cu-
lumbia blood agar plates were counted after a 3-day incubation
for P. intermedia and after a 5-day incubation for B. forsythus
(37°C, anaerobically). The detection limit was determined by
using known numbers of bacteria diluted either in reduced
transport fluid or in subgingival plaque samples from five
healthy volunteers previously checked with the multiplex PCR
to be free of B. forsythus and P. intermedia.

In the 1:1 mixture of pure cultures of B. forsythus and P. interme-
dia, the multiplex PCR detected between 50 and 500
CFU of each species. In contrast, the detection limit was slight-
ly increased for the spiked subgingival plaque samples and
found to range between 100 and 1,000 CFU. By decreasing
the annealing temperature from 55 to 52°C and increasing the
concentration of magnesium (1.5 to 4.5 mM), the sensitivity of
our procedure could be increased to a single cell but resulted
in weak cross-reactivity and nonspecific amplification bands
(data not shown). Since subgingival plaque is a mixed commu-

Sample processing for PCR. Three different methods of
sample preparation for PCR were tested with pure cultures, 12
clinical specimens (for each method, one subgingival plaque
sample and one swab sample each from the tonsils, the buccal
mucosa, and the dorsum of the tongue), and spiked subgingival
plaque from healthy volunteers.

(i) Boiling. Deep-frozen 250-μl suspensions (bacterial cul-
tures or patient specimens) were incubated for 10 min at 37°C.
Four glass beads (2 mm in diameter) were then added, the
samples were vortexed for 20 s and centrifuged, the super-
natant was removed, and the pellet was resuspended in 100
μl of distilled water. After an additional vortexing-and-centrifuga-
tion step, the pellet was resuspended in 500
μl of distilled water. After an additional vortexing-and-centrifuga-
tion step, the pellet was resuspended in 100
μl of distilled water and the suspension was heated for 10 min at 94°C with a
thermocycler. The vials were then stored for 5 min on ice and
centrifuged, and 5-μl aliquots of the supernatant were further
used in the PCR assay.

It has recently been reported that Chelex 100 resin (Bio-Rad
Laboratories, Hercules, Calif.) (9) processing of oral speci-
mens prior to boiling most effectively decreases PCR inhibition
and thus increases sensitivity (21). Therefore, we performed an
additional experiment by using subgingival plaque and mucosal
swabs taken from three additional patients, spiking the samples
with B. forsythus and P. intermedia, and processing them with
Chelex 100.

(ii) Lysozyme-phenol protocol. A second way of isolation
was performed by using a previously described protocol (6).
After lysozyme-phenol processing of 250-μl suspensions in ac-
cordance with this protocol, the aqueous phase was adjusted to
2.5 M ammonium acetate and the nucleic acid was precipitated
with 2.5 volumes of ice-cold 70% ethanol. After
centrifugation, the nucleic acid pellet was washed with 250 μl
of 70% ice-cold ethanol and dissolved in 500 μl of distilled
water.

(iii) QIAamp Tissue Kit protocol. Suspensions of 250 μl
were pelleted by centrifugation for 10 min at 5,000 × g, resus-
pended in 180 μl of enzyme incubation buffer (20-μg/ml ly-
sozyme, 20 mM Tris-HCl [pH 8.0], 2 mM EDTA, 1.2% Triton
X-100), and incubated for 30 min at 37°C. A 20-μl volume of
proteinase K stock solution (20 μg/ml) was added, and the
sample was mixed by vortexing and incubated at 55°C in a
shaking water bath until it was completely lysed. Afterwards,
isolation was performed as recommended by the manufacturer
(QIAGEN GmbH, Hilden, Germany).

PCR amplification. PCR amplification was carried out in a
volume of 100 μl containing 1× PCR buffer, 1.5 mM MgCl2, 2
U of Taq polymerase (Boehringer, Mannheim, Germany), 0.2
mM each deoxyribonucleoside triphosphate (Boehringer), 5 pmol
of a universal 16S rDNA forward primer (pA: 5′ AGA GTT
TGA TCC TGG CTC CAG 3′) (10), 5 pmol of either of the two
species-specific primers (BFV530 5′ GTA GAG CTA ACA
CTA TAT CGC AAA CTC CTA 3′) for detection of B. forsy-
thus (14) or Pi 5′ GTT GGC TCG ACT CAA GTC CGC C
3′) for detection of P. intermedia (7) or a universal reverse
primer (pH°: 5′ AAG GAG GTG ATC CAG CCG CA 3′)
and 5 μl of either the template (approximately 100 ng) or
the reference (100 ng) nucleic acids. All oligonucleotides were
synthesized on a DNA synthesizer (OLIGO 1000; Beckman,
Munich, Germany). Amplification was performed by using 30
cycles of the following temperature profile: denaturation for 1
min at 94°C, annealing for 1 min at 55°C, and elongation for 2.5
min at 72°C. After the 30 cycles, a final elongation step of 5 min
at 72°C was added. Amplification products (aliquots of 10 μl)
were separated electrophoretically on a 2% agarose gel
(Merek, Darmstadt, Germany) in 1× TPE (80 mM Tris-phos-
phate, 2 mM EDTA [pH 7.5]). From two independent data-
bases (23a, 24a), the 16S rDNA sequences and the annealing
sites of primers were determined for both test species. The
search indicated amplicon sizes of 660 bp for P. intermedia and
840 bp for B. forsythus.

Specificity and sensitivity of the PCR. The specificity of the
PCR was evaluated by testing 6 B. forsythus and 10 P. interme-
dia strains, as well as 23 representatives of closely or more
distantly related species (aliquots of 100 ng of nucleic acid).
Amplicons appearing to be of the expected sizes (P. intermedia,
660 bp; B. forsythus, 840 bp) were found with all of the strains
tested. Neither of these two PCR products or other PCR bands
were pelleted by centrifugation for 10 min at 5,000 × g, and
were separated electrophoretically on a 2% agarose gel
and after a 5-day incubation for B. forsythus (37°C, anaerobically). The detection limit was determined by
using known numbers of bacteria diluted either in reduced
transport fluid or in subgingival plaque samples from five
healthy volunteers previously checked with the multiplex PCR
to be free of B. forsythus and P. intermedia.
in order to maintain the highest possible specificity. Lack of control lacking template DNA.

control with 100 ng each of mucosa) from four patients (no. 1 to 4 in Table 1). Lanes: M, AmpliSize standard for a PCR. Therefore, 55°C and 1.5 mM MgCl2 were employed ininity of different species, high specificity is especially important for a PCR. Therefore, 55°C and 1.5 mM MgCl2 were employed in order to maintain the highest possible specificity.

Detection of B. forsythus and P. intermedia in clinical specimens. The yield of nucleic acids isolated from 12 clinical samples by the three methods described was between 10 μg (boiling) and 50 μg (QIAamp Tissue Kit). It was calculated that DNA extraction from the pure bacterial cells and spiked plaque also yielded similar amounts of nucleic acids. Because isolating pure DNA with the QIAamp Tissue Kit is expensive, boiling was selected as the method of choice to process the remaining 140 specimens.

A representative multiplex PCR result for clinical samples is demonstrated in Fig. 1. The following pattern (Table 1) was found when the 152 clinical samples from different origins were analyzed for the presence of B. forsythus and/or P. intermedia. (i) In 5 of the subgingival plaque samples (13.2%), both species were present, 11 samples (28.9%) demonstrated B. forsythus only, and 1 sample (2.6%) contained P. intermedia only. (ii) Four (10.5%) of 38 tonsil swabs were positive for P. intermedia only, and one specimen harbored both B. forsythus and P. intermedia. (iii) Of the buccal mucosa swab samples, one was positive for both B. forsythus and P. intermedia, six (15.8%) were positive for P. intermedia, and four (10.5%) were positive for B. forsythus. (iv) Finally, 10 (26.3%) of the tongue swabs were positive for P. intermedia but none was positive for B. forsythus. None of the periodontopathogenic species assessed were detected in 21 of 38 subgingival plaque samples, 33 of 38 tonsil swabs, 27 of 38 buccal epithelial swabs, and 26 of 38 tongue swabs. Because these samples contained approximately 30 to 100 different species, this finding supports the specificity of our multiplex PCR. To exclude inhibitory compounds as a principal reason for a negative result, a universal 16S rDNA-directed PCR combining universal primers pA and pH° was used and found to be positive for all culture and clinical specimens. However, it is possible that for some samples with multiplex PCR-negative results, a low concentration of target bacteria (<500 cells) and/or inhibitory compounds, such as cations, caused a false-negative result (1, 18). Since Chelex 100 processing of 12 additional samples resulted in an enhanced sensitivity of 50 CFU/clinical specimen without loss of specificity, we recommend this procedure prior to boiling for future application of the described multiplex PCR.

Previous studies (14, 23) have focused on subgingival plaque as a likely primary habitat of B. forsythus. In the present study, we investigated the localization of this obligate anaerobic, fastidious species in ecological niches of the oral cavity other than subgingival plaque. Interestingly, the finding that 13.1% of all buccal mucosa swabs were positive for B. forsythus indicates that the buccal membrane is one of its habitats in the oral

<table>
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<tr>
<th>Patient no.</th>
<th>B. forsythus without P. intermedia</th>
<th>P. intermedia without B. forsythus</th>
<th>B. forsythus and P. intermedia</th>
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* Positive sites in parentheses: P, subgingival plaque; T, tonsil swabs; B, buccal membrane swabs; Z, tongue mucosal swabs.
cavity. In contrast, the prevalence of *B. forsythus* on the tonsil or tongue mucosa seems to be rather low, even in patients with untreated periodontitis. Surprisingly, the prevalence of *P. intermedia* among the specimens tested was highest on the dorsum of the tongue (26.3%).

In conclusion, multiplex PCRs may rapidly detect relevant numbers of putative periodontal pathogens cost effectively in clinical specimens. To assist in specific treatment planning, microbiological diagnosis of periodontal infections is increasingly needed. Clinical laboratories which test samples sent in by mail have recently been established, and tests which can be performed in a dentist’s office have also been introduced (27). These tests depend mainly on dot blot hybridization assays using both genomic or oligonucleotide DNA probes.

The method described in this report for the simultaneous detection of *B. forsythus* and *P. intermedia* may assist in selecting adjunctive antibiotics for the treatment of aggressive periodontal diseases. Whether the detection of putative periodontal pathogens may also be useful in assessing the risk or progression of periodontal disease onset is unclear and should be established.

This work was supported by a grant of the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (BMBF no. 01KI9710/5) of Germany.

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