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PCR Assay for Species-Specific Identification of *Bacteroides thetaiotaomicron*

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***Bacteroides thetaiotaomicron* is the second most frequently encountered species of the anaerobes isolated from clinical specimens. We developed a PCR-based assay for the rapid identification of *B. thetaiotaomicron*. Specific primers were based on shared amplicons of about 1.2 kb generated from *B. thetaiotaomicron* by randomly amplified polymorphic DNA. This 1.2-kb fragment was sequenced and then used to design a set of PCR amplification primers. This PCR generated an amplification product of 721 bp, which was unique to all 65 isolates of *B. thetaiotaomicron* tested. There was no amplification with isolates of other bacterial species. Restriction enzyme digestion of the amplification product and dot blot hybridization further verified the specificity of the assay. These results suggest that this PCR assay targets a nucleotide sequence that is strongly conserved in *B. thetaiotaomicron*. This simple and rapid PCR assay provides a rapid and accurate method for identification of *B. thetaiotaomicron* and shows promise for the detection of *B. thetaiotaomicron* in clinical samples.**

Members of the *Bacteroides fragilis* group, which are bile-resistant, anaerobic, gram-negative rods, are opportunistic pathogens commonly recovered from patients with peritonitis, septicemia, and wound infections. Of the greatest clinical importance within this group are *B. fragilis* and *B. thetaiotaomicron*. *B. thetaiotaomicron* is the second most commonly encountered anaerobic gram-negative bacillus (1, 5; I. Brook, Letter, J. Antimicrob. Chemother. **25:**473–474, 1990). Although it is part of the indigenous microflora of the gastrointestinal tract, it is also associated with infection, such as intra-abdominal sepsis and bacteremia, and is resistant to many antimicrobial agents (1, 5; Brook, Letter). It has been reported that *B. fragilis* group bacteremia contributes significantly to morbidity and mortality (15). In addition, *B. thetaiotaomicron* has been reported to be more resistant to cephalosporins and clindamycin than *B. fragilis* (1, 5, 15, 16, 18). These clinical characteristics of *B. thetaiotaomicron* infection increase the need for rapid and accurate identification of the infection in clinical specimens and for immediate and effective management of infected patients.

Conventional biochemical identification of *Bacteroides* species is complicated and time-consuming. Species identification often requires 3 to 7 days and sometimes is inconclusive (19). The automated methods currently used are sometimes unreliable at identifying *B. thetaiotaomicron* clinical isolates or other species (2, 3). The phenotypic and biochemical similarity of closely related species makes it difficult to discriminate them. Precise identification is not always possible. This difficulty in correctly identifying the *B. thetaiotaomicron* strains may subsequently result in inappropriate antibiotic therapy and should be of concern to clinical laboratories. Thus, a more specific and rapid assay is needed for the identification of *B. thetaiotaomicron*. In recent years, PCR methods have been developed to identify a variety of microorganisms including anaerobes. Yamashita et al. used the glutamine synthetase gene as a target for amplification of *B. fragilis* (20). Kuwahara et al. reported the detection of *B. fragilis* by PCR amplification of the neuraminidase-encoding gene (11). Genotypic methods for the detection of *B. thetaiotaomicron* using PCR fingerprinting or PCR hybridization have been reported (4, 10). Kreader described the use of a PCR hybridization assay based on 16S rRNA sequences to identify *B. thetaiotaomicron* and other species from fecal extracts (10). However, it is difficult to adapt this test for use in clinical laboratories because of its complexity. The aim of the present study was to develop a simple and species-specific PCR assay for the identification of *B. thetaiotaomicron*.

Bacterial strains. Twenty-seven reference strains obtained from the American Type Culture Collection, Rockville, Md., were used in this study. The PCR assay primers were based on the reference strain, *B. thetaiotaomicron* ATCC 29741. Other species representing 11 gram-positive and 15 gram-negative bacterial species were used as negative controls (Table 1). An additional 65 clinical isolates of *B. thetaiotaomicron* and 40 clinical isolates of *Bacteroides* species were also tested. All of the clinical isolates were collected from the Bacteriology Laboratory, National Taiwan University Hospital, a 2,000-bed teaching hospital in northern Taiwan. The *Bacteroides* species was identified by the Presumpto Plates method and sugar fermentation tests as previously described (19).

RAPD fingerprint among *Bacteroides* species. Randomly amplified polymorphic DNA (RAPD) patterns of tested strains were determined by means of arbitrarily primed PCR as described in our previous report (8). The arbitrary oligonucleotide primer OPH-9, 5′-TGTAGCTGGG-3′ (Operon Technologies, Alameda, Calif.), was used. Amplification products were analyzed by electrophoresis on a 1.5% agarose gel (FMC Bioproducts, Rockland, Me.). The RAPD fingerprint patterns obtained with the primer OPH-9 for *B. thetaiotaomicron* and

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TABLE 1. Bacteria used as negative controls in this study

Bacterial strain or isolate
Bacteroides ovatus ATCC 8483 and 6 clinical isolates
Bacteroides fragilis ATCC 25285 and 10 clinical isolates
Bacteroides distasonis ATCC 8503 and 5 clinical isolates
Bacteroides uniformis ATCC 8492 and 8 clinical isolates
Bacteroides vulgatus ATCC 8482 and 5 clinical isolates
Bacteroides caccae ATCC 43185 and 6 clinical isolates
Bacteroides stercoris ATCC 43183
Clostridium perfringens ATCC 3624
Clostridium difficile ATCC 9689
Eubacterium lentum ATCC 43055
Fusobacterium nucleatum ATCC 25586
Fusobacterium varium ATCC 8501
Prevotella intermedia ATCC 25611
Peptostreptococcus anaerobius ATCC 27337
Propionibacterium acnes ATCC 6919
Veillonella parvula ATCC 10790
Staphylococcus aureus ATCC 29213
Staphylococcus epidermidis ATCC 14990
Streptococcus bovis ATCC 9809
Streptococcus anginosus ATCC 33397
Streptococcus intermedius ATCC 27335
Enterococcus faecalis ATCC 29212
Escherichia coli ATCC 25922
Aeromonas hydrophila ATCC 7965
Acinetobacter baumanii ATCC 19606
Pseudomonas aeruginosa ATCC 27853

other *Bacteroides* species are shown in Fig. 1. One shared band of about 1.2 kb was found in the RAPD fingerprints of all five of the *B. thetaiotaomicron* isolates tested. This fragment was absent from the RAPD fingerprints of the other six species (Fig. 1).

Extraction and sequencing of DNA from a RAPD fingerprint band. This 1.2-kb fragment was excised, purified, and sequenced. The selected amplicon was excised from the RAPD gel and was eluted by using the Gene-Clean kit (Bio 101, Inc., La Jolla, Calif.). The DNA fragment was cloned into the vector using the TA cloning kit and transformed into *Escherichia coli*. Following thermal cycling of the sequencing reactions with fluorescent-dye-labeled primers or terminators, the nucleotide sequence was determined on an Automated DNA Sequencer (ABI prism 377A DNA sequencer; Applied Biosystems Division, Perkin-Elmer Corp., Foster City, Calif.) The partial sequence of the 1.2-kb fragment is shown in Fig. 2. No significant

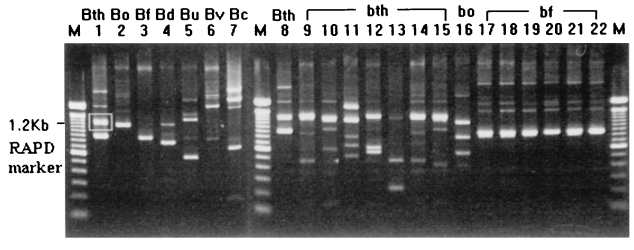

FIG. 1. RAPD patterns generated from *B. thetaiotaomicron* and other *Bacteroides* species. RAPD patterns were obtained by AP-PCR amplification of genomic DNA with the primer OPA-09. Lanes M contain 100-bp DNA ladder (Gibco BRL). Lanes 1 and 8, *B. thetaiotaomicron* (Bth) ATCC 29741; lane 2, *B. ovatus* (Bo) ATCC 8483; lane 3, *B. fragilis* (Bf) ATCC 25285; lane 4, *B. distasonis* (Bd) ATCC 8503; lane 5, *B. uniformis* (Bu) ATCC 8492; lane 6, *B. vulgatus* (Bv) ATCC 8482; lane 7, *B. caccae* (Bc) ATCC 43185; lanes 9 to 15, *B. thetaiotaomicron* (bth) clinical isolates; lane 16, *B. ovatus* (bo) clinical isolate; lanes 17 to 22, *B. fragilis* (bf) clinical isolates. In lane 1, the 1.2-kb amplicon shared by *B. thetaiotaomicron* strains is indicated by the white box around the band.

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ACCATTCGGG TTGGAGTTTT ACTTTGAATG GACAATCTAG CGACTTGTAT    50
          → BTH-F primer
GATTATGTTC CGGATGGCAG CATACTTGAT ATCGATTTGG GAAAACAAGA   100

GAATATAAAA ACTATTGCTT TACATTTCTA TGAATGGTTT TATTCTTCGG   150

AAAGTGCAAG TATAGCGATA AGTAATGATG GAGAGAAATA TGAGGATCTT   200

GGTGTAGCTT CAGGATTTGC AAATAAGACA AGTTACATTC TTCTTCTTGT   250

GGCTAAACAA GCGCAATATA TAAGAGTAAC TTTCCATGGA GCTCTTTATT   300

ATTCTCCTTA TATTAATACT GTAGGTATTT ATACTGAAAC TGAATAAAAA   350

ATGGCCCAAG TTTATTACTG AATGAGCTTG GGCTATTTGC TGTTTATATC   400

ATGGTGTGAT CAGTTCTAAG TTTTCTTTGT TAATAAAACT TAGAACTGAT   450

TTTTTTCTTG AGTATCTTAT ATTTTTGGAT TGTATTATCT AAAAACTAAT   500

TAAAGGAAAT GCTATGAGAA TCATTCGCCG TATTATTTTT CAGCTGCTTT   550

GTTTGTTGGG AGCATGTTGT TATATTCCTG CAACAGCACA AGTCGTTTTA   600

GTTGATAATG GAAAAACTAA ATCCAGGATT ATTCTATCAG AAAATGACCA   650

GATTAATCAA ATATCAGCAA ATTTATTTCA ATTGTTCCTT CAGAGAATTT   700

CAGGTTGTAC ATTTCCCATT GTAAAAGGGC AGAATGCAAA AAAAGGAGAT   750
                BTH-R primer ←
ATTATAATCA GTAGCAAAAC CCCAGCTACA                         780
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FIG. 2. Partial sequences of the specific RAPD marker. The PCR primers are underlined.

homologies were found between the sequences of this fragment and those of other genes found in the GenBank database.

Development of PCR. The primer pair BTH-F (5′-TGGAG TTTTACTTTGAATGGAC-3′) and BTH-R (5′-CTGCCCTT TTACAATGGG-3′) based on the sequences of the 1.2-kb fragment were designed to generate a 721-bp product upon PCR amplification of DNA from *B. thetaiotaomicron* ATCC 29741 (Fig. 2). The amplification reaction mixtures contained 50 μl of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 1 U of *Taq* polymerase (Perkin-Elmer, Norwalk, Conn.), 200 μM each of the four deoxynucleotide triphosphates (dATP, dCTP, dGTP, and dTTP; Perkin-Elmer), 50 pmol of each primer, and 2 μl of DNA sample. PCR was performed in a DNA thermal cycler (MJ Research, Inc., Watertown, Mass.). A total of 35 cycles of PCR were done, with 1 cycle consisting of denaturation (94°C, 1 min), annealing (56°C, 1 min), and extension (72°C, 1 min) steps followed by a final extension step (72°C, 7 min). The PCR amplification products were analyzed by agarose gel electrophoresis in 1% agarose (FMC BioProducts) and stained with ethidium bromide. A visible band of the appropriate size (721 bp) was considered a positive reaction. The conserved sequence was further analyzed by digestion of the PCR products with the restriction enzymes *Hae*III and *Hin*fI (Gibco BRL, Gaithersburg, Md.). The DNA fragments were run on 1.8% agarose gels (FMC BioProducts).

After PCR, the 721-bp DNA product was obtained only from isolates of *B. thetaiotaomicron*. No amplification was detected from other species (Fig. 3).

Specificity and sensitivity of PCR assays. The specificity and sensitivity were further determined by testing a total of 65 isolates of *B. thetaiotaomicron* and 40 isolates of other species.

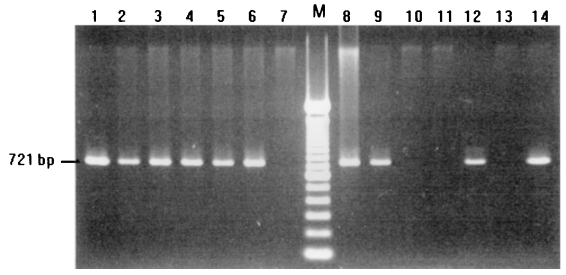

FIG. 3. Specificity of PCR amplification. Specific amplification of the 721-bp DNA fragment was detected only in *B. thetaiotaomicron* isolates. Lane, M, 100-bp DNA ladder (Gibco BRL); lanes 1 to 6, 8, 9, 12, and 14, *B. thetaiotaomicron* isolates; lane 7, *B. fragilis*; lane 10, *B. distasonis*; lane 11, *B. uniformis*; lane 13, *B. vulgatus*.

All 65 clinical isolates identified as *B. thetaiotaomicron* by conventional methods yielded the 721-bp amplicon. Moreover, the PCR amplification products from *B. thetaiotaomicron* isolates all revealed identical *Hae*III and *Hin*fI restriction patterns (Fig. 4). No amplification products were detected with 40 isolates of all other species. The sensitivity of PCR was determined by testing with serial dilutions of the DNA sample. The level of sensitivity indicates that several copies of the target DNA are needed for detection.

Dot blot assays. To confirm the specificity of this PCR assay, the 721-bp PCR product was labeled with digoxigenin and then used for hybridization to genomic DNA from various organisms. Probes were produced with the PCR method described above and simultaneously labeled by incorporation of digoxigenin-11-dUTP (Boehringer Mannheim, Mannheim, Germany). For each strain tested, 300 ng of chromosomal DNA was denatured by heating at 96°C for 10 min and spotted onto Hybond-N nylon membranes (Amersham). DNA was then fixed onto the filter by UV treatment at an intensity of 120 mJ/cm^2 for 3 min on a UV Crosslinker. Both the prehybridization and hybridization temperatures were 61°C. All filters were prehybridized for 1 h in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Hybridization was performed overnight with heat-denatured probe. Detection was performed by using an antidigoxigenin antibody conjugated to alkaline phosphatase as a substrate according to the manufacturer's instructions. Only DNA from *B. thetaiotaomicron* showed a strong hybridization signal. No hybridization signal was detected by dot blot analysis of DNA from non-*B. thetaiotaomicron* strains (Fig. 5).

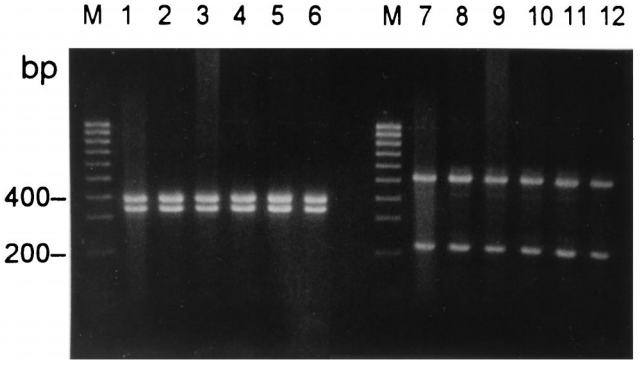

FIG. 4. PCR-restriction fragment length polymorphism analysis of *B. thetaiotaomicron*. The PCR products with primers BTH-F and BTH-R from *B. thetaiotaomicron* isolates were digested with restriction enzymes *Hae*III (lanes 1 to 6) and *Hin*fI (lanes 7 to 12). Lanes M contain 100-bp DNA ladder (Gibco BRL).

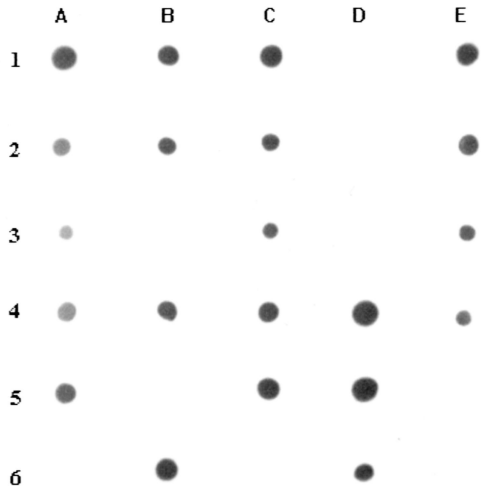

FIG. 5. Dot blot hybridization showing the specificity of PCR amplification. All positive hybridization reactions (indicated by the presence of a hybridization signal) were obtained with *B. thetaiotaomicron* isolates. All negative reactions (no hybridization signal) were from other species as follows: *B. ovatus* (1D), *B. uniformis* (2D), *B. distasonis* (3B), *B. fragilis* (3D), *B. vulgatus* (5B), *Staphylococcus aureus* (5E), *Streptococcus bovis* (6A), *Enterococcus faecalis* (6C), and *Escherichia coli* (6E).

B. thetaiotaomicron isolates are commonly found with serious extraintestinal tract infections and are usually more resistant to antimicrobial agents than *B. fragilis*. Accurate identification of *Bacteroides* species is often problematic. Conventional identification protocols are usually laborious and time-consuming (19). It is sometimes difficult to differentiate among indole-positive *Bacteroides* species. For final identification, it was necessary to perform additional tests (production of acid from arabinose, trehalose, and xylane) to discriminate between *B. fragilis*, *B. thetaiotaomicron*, *B. ovatus*, and *B. uniformis*. It has been reported that misidentification of most *Bacteroides* species often involves *B. thetaiotaomicron* (2, 3). For such isolates, up to 48 h can be required for further identification assays. Phenotypic differentiation between *B. thetaiotaomicron* and *B. ovatus* is often difficult in a clinical laboratory setting (19). *B. thetaiotaomicron* is phylogenetically very close to *B. ovatus*. Although 16S rRNA has been widely used as a target for PCR primers or probes for the identification of microorganisms (6), the assay for *B. thetaiotaomicron* is less selective, since both *B. thetaiotaomicron* and *B. ovatus* were detected when the 16S rRNA probe of *B. thetaiotaomicron* was used (10). In fact, on the basis of 16S rRNA sequence comparison, these two species exhibited 96.9% sequence homology (13). Therefore, the development of rapid and sensitive DNA-based assays which are applicable for the direct detection of *B. thetaiotaomicron* may improve the rapidity and accuracy of the diagnosis of infections.

In the present study, we have developed a rapid PCR-based assay to improve the identification of *B. thetaiotaomicron*. Initially, RAPD analysis was performed to compare the patterns generated from *Bacteroides* species. A conserved fragment was observed with *B. thetaiotaomicron* isolates. Subsequently, the primers were designed based on the sequences of this fragment and a PCR assay was set up. RAPD analysis has been used mostly for intraspecies discrimination in epidemiological studies (8, 9). However, PCR assays based on the primers designed

from conserved fragments generated by RAPD have been previously reported (7, 12, 14, 17). For some organisms, such as *Prevotella*, *Porphyromonas*, *Legionella*, and *Candida* species, this method has been successfully applied for species identification (7, 12, 14, 17). This strategy can be seen as a universal method for designing detection assays without the need for prior knowledge of the genetic characteristics of the target species.

The species specificity of this PCR assay was further tested on 65 clinical isolates identified as *B. thetaiotaomicron* by conventional methods and 11 species of gram-positive and 15 species of gram-negative clinically important bacteria tested. The specific 721-bp amplicon can be obtained only from all *B. thetaiotaomicron* isolates, not from other species. The sensitivity of this PCR assay indicates that only several copies of the target DNA are needed for detection. The sensitivity level should be sufficient for the direct detection of *B. thetaiotaomicron* in clinical specimens.

In order to ensure the specificity of amplification and also examine the intraspecies heterogeneity, restriction digestion was performed. All of the *B. thetaiotaomicron* clinical isolates displayed identical *Hae*III and *Hin*fI restriction patterns. Therefore, no intraspecies heterogeneity was found. The conservation of target sequences was also confirmed by dot blot hybridization. Under these conditions, only DNA from *B. thetaiotaomicron* showed a strong hybridization signal. No hybridization signal was detected by dot blot analysis of DNA from non-*B. thetaiotaomicron* strains. This assay shows promise for the detection of *B. thetaiotaomicron* from clinical specimens.

The PCR-based diagnostic assay described in this study targets a nucleotide sequence that is conserved in *B. thetaiotaomicron*. No significant homologies were found between the sequence of the 721-bp amplification product and those of other genes found in the GenBank database. No probe is needed in this assay. The simple and rapid PCR assay offers an alternative to currently used methods and may lead to the early diagnosis of *B. thetaiotaomicron* infections.

Nucleotide sequence accession number. The partial sequence of the 1.2-kb fragment from the RAPD fingerprints of *B. thetaiotaomicron* isolates was deposited in GenBank and assigned accession no. AF182955.

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