Detection of *Bacteroides fragilis* in Clinical Specimens by PCR

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Received 25 August 1993/Returned for modification 28 September 1993/Accepted 2 December 1993

The direct detection of *Bacteroides fragilis* strains was examined by using the PCR method for amplifying a specific fragment of the glutamine synthetase gene from *B. fragilis*. By this method, all five *B. fragilis* strains tested were detected, but DNAs from anaerobic bacteria of 24 other species tested, from aerobic bacteria of 12 species tested, and from human leukocytes were not amplified. Using the nested PCR method, we were able to detect as little as one bacterial cell or 100 fg of chromosomal DNA of *B. fragilis*. A total of 39 clinical specimens, which consisted of 19 bronchial aspirates, 10 percutaneous lung aspirates, 2 tracheal aspirates, 6 pleural fluid specimens, and 2 pus specimens, were tested. All four culture-positive samples, of which two were bronchial aspirates, one was pleural fluid, and one was pus, were positive by PCR. Among 35 culture-negative samples, 2 bronchial aspirates were positive by PCR. One was from a patient whose two previous specimens were positive by both culture and PCR. It had been submitted for culture several hours after collection, and clindamycin had been administered to the patient before collection of the specimen. The other bronchial aspirate positive by PCR was from a pneumonia patient who had also been administered clindamycin. We believe that *B. fragilis* was present in these two specimens but that either it was dead, it was below the level detectable by culture, or the process of anaerobic culture was unsuccessful. Thus, the PCR method may be considered useful for the sensitive and rapid detection of anaerobes in clinical specimens.

Anaerobes are known to be causative organisms of various infectious diseases in humans (4, 8), but the need for special techniques and equipment to culture these organisms has delayed improvement in detection of anaerobes from clinical specimens (13). It is more difficult to isolate anaerobes from lower respiratory tract infections, in particular, because anaerobes exist within the oral cavity as normal flora (13). Therefore, appropriate specimens for anaerobic culture need to be taken by special techniques, such as tracheal aspiration, percutaneous lung aspiration, bronchoscopy aspiration with a protected catheter brush (PCB), and the like, which were developed in order to avoid oropharyngeal contamination (1–4). Since anaerobic conditions are difficult to maintain when a small quantity of specimen is obtained and since the lidocaine used for local anesthesia in bronchoscopic examination has antibacterial effects (1, 2, 4), the successful culture of anaerobes is difficult. Additionally, conventional methods for identification of anaerobes by biochemical tests or gas-liquid chromatography are troublesome and time-consuming, and commercial kits for rapid identification are less accurate than conventional methods (13).

The anaerobes frequently isolated from human clinical specimens are *Bacteroides fragilis*, *Prevotella melaninogenic* *, Fusobacterium nucleatum*, and *Peptostreptococcus* spp. (Weakly saccharolytic *Bacteroides* spp. have now been reclassified in the genus *Prevotella* [8].) *B. fragilis* is particularly important because of its pathogenicity, frequency, and resistance to many commonly used antimicrobial agents (8).

In order to rapidly detect and/or identify *B. fragilis* and other *Bacteroides* spp., the use of various methods, such as immunofluorescence (6, 21), DNA probes (10, 15), and rRNA restriction fragment length polymorphisms (19), has been described. However, each of these methods has disadvantages in specificity or sensitivity. In recent years, the PCR method (16), which can amplify a specific gene fragment rapidly in vitro, has been developed and come into wide use. In the field of clinical bacteriology, this method is very useful to detect organisms which are difficult to culture, for example, *Mycobacterium tuberculosis* (12, 14, 18), *Legionella pneumophila* (7, 20), and the like. Furthermore, a nested PCR method which is composed of a two-step PCR can enhance sensitivity more than the single-step PCR (11, 12, 14). Thus, the PCR method can detect a target organism directly from clinical specimens without culture. In this study, we used the nested PCR method to detect *B. fragilis* in clinical specimens obtained from patients with anaerobic infections, mainly lower respiratory tract infections.

**MATERIALS AND METHODS**

**Bacterial strains.** The organisms used to evaluate the specificity of the PCR method for detection of *B. fragilis* are as follows. For anaerobic bacteria, the following 29 strains, most of which were kindly supplied by the Institute of Anaerobic Bacteriology, Gifu University School of Medicine, were tested: *B. fragilis* (ATCC 25285 and four clinical isolates), *Bacteroides caccae* (GAI-973173), *Bacteroides distasonis* (GAI-5462), *Bacteroides eggerthii* (GAI-5478), *Bacteroides merdae* (GAI-93174), *Bacteroides ovatus* (GAI-5630), *Bacteroides stercoris* (GAI-93175), *Bacteroides thetaiotaomicron* (ATCC 29741), *Bacteroides uniformis* (GAI-5466), *Bacteroides vulgatus* (GAI-5460), *Bacteroides ureolyticus* (GAI-5544), *Prevotella bivia* (GAI-5518), *Prevotella intermedia* (GAI-5592), *Prevotella oralis* (GAI-7801), *Prevotella oris* (GAI-7508), *P. melaninogenic* *a* *(clinical isolate)*, *Porphyromonas gingivalis* (GAI-7802), *F. nucleatum* (GAI-5464), *Fusobacterium varium* (GAI-5566), *Fusobacterium necrophorum* (GAI-5634), *Peptostreptococcus magnus* (GAI-5600), *Peptostreptococcus asaccharolyticus*.
patients with pyothrax. Both of two samples of pus were obtained from patients with subcutaneous abscesses. Each sample was tested by Gram stain, aerobic and anaerobic culture, and nested PCR examination.

**Bacteriologic processing.** The specimens were plated onto the following four kinds of media for anaerobic culture (Kyokuto Pharmaceutical Co., Tokyo, Japan): brucella HK agar with hemolyzed rabbit blood and defibrinated sheep blood, phenylethyl alcohol brucella HK agar with hemolyzed rabbit blood, paromomycin-vancomycin brucella HK agar with hemolyzed rabbit blood, and bacteroides bile esculin agar. The specimens generally were plated immediately after collection, and then the media were placed in GasPak Pouches (BBL) to put them under anaerobic conditions. They were incubated at 37°C for more than 2 days and less than 7 days. An anaerobic chamber (Forma Scientific Inc.) or anaerobic jars (AnaerO-Pack; Mitsubishi Gas Inc., Tokyo, Japan) were used for subculture. Anaerobes isolated by this process were identified by using RapID-ANA (Innovative Diagnostic Systems, Inc., Atlanta, Ga.). As media for aerobes and fungi, sheep blood agar (BBL), chocolate agar (BBL), bromthymol blue lactose agar (Nissui Pharmaceutical Co.), and Inhibitory Mold Agar (BBL) were used.

DNA extraction from bacterial strains. A large number of colonies obtained from each bacterial strain on agar were suspended in 400 μl of TE buffer (10 mM Tris hydrochloride [pH 8], 1 mM EDTA). Each bacterial strain cultured in broth was centrifuged at 10,000 × g for 10 min, and the pellet was resuspended in 400 μl of TE buffer. Suspensions of gram-negative bacteria were incubated with lysozyme (to a final concentration of 1 mg/ml), and suspensions of gram-positive bacteria were incubated with mutanolysin (to a final concentration of 125 U/ml) at 37°C for 60 min. Proteinase K and sodium dodecyl sulfate (SDS) were added to each suspension to final concentrations of 1 mg/ml and 1%, respectively, and each mixture was incubated at 60°C for 4 h. For the extraction of nucleic acids, an equal volume of phenol-chloroformisoamyl alcohol (25:24:1) was added, each mixture was centrifuged at 10,000 × g for 2 min, and then the supernatant was transferred to a fresh tube. This procedure was repeated twice, and then the same procedure with an equal volume of chloroform-isoamyl alcohol (24:1) was performed once. The DNA was precipitated with 1/10 volume of 3 M sodium acetate and 2 volumes of ice-cold absolute ethanol at −80°C for 30 min and then centrifuged at 10,000 × g for 15 min at 0°C. The air-dried pellet was resuspended in double-distilled water and diluted to 10 ng/μl. The quantity of DNA in each sample was measured with a fluorometer (model 450; Sequoia-Turner Corporation). Serial dilutions containing DNA of *B. fragilis* in amounts from 10 ng to 10 fg were prepared to calculate the detection limit according to the DNA quantity.

DNA extraction from clinical specimens. When the specimens were purulent, they were incubated with dithiothreitol (to a final concentration of 1 mg/ml) at 37°C for 30 min at the beginning of the procedure. All samples were centrifuged at 10,000 × g for 10 min, and each pellet was resuspended in 400 μl of TE buffer. They were incubated with lysozyme (1 mg/ml) at 37°C for 60 min and then with proteinase K (1 mg/ml) and SDS (1%) at 60°C for more than 4 h. Next, DNA was extracted by phenol and chloroform treatment and recovered by ethanol precipitation as described above. The extracted DNA was resuspended in 100 μl of distilled water, 10 μl of which was used for PCR.

**Synthetic oligonucleotides.** Two sets of primers encoding the glutamine synthetase gene (reported by Hill et al. [5]) were synthesized and used for the nested PCR. The sequence of the

**TABLE 1. Detection of *B. fragilis* in clinical specimens**

<table>
<thead>
<tr>
<th>Specimen</th>
<th>PCR positive</th>
<th>PCR negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Culture positive</td>
<td>Culture negative</td>
<td>Culture positive</td>
</tr>
<tr>
<td>Bronchial aspirate</td>
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<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Cannula</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PCB</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Percutaneous lung</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Transtrachial</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Aspirate</td>
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<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Pleural fluid</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>4</td>
<td>2</td>
<td>0</td>
</tr>
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</table>

* * Among these specimens, nine were obtained from patients with pneumonia and seven were from patients with lung abscess.

* * Among these specimens, three were obtained from patients with pneumonia and seven were from patients with lung abscess.

(GAI-5534), *Peptostreptococcus anaerobius* (GAI-5598), *Peptostreptococcus micros* (GAI-5540), and *Veillonella parvula* (GAI-5602). For aerobic bacteria, the following 12 strains were tested: *Staphylococcus aureus* (ATCC 25923), *Streptococcus pneumoniae* (clinical isolate), *Streptococcus intermedius* (ATCC 27735), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (clinical isolate), *Enterobacter cloacae* (ATCC 23355), *Serratia marcescens* (ATCC 8100), *Proteus mirabilis* (clinical isolate), *Pseudomonas aeruginosa* (ATCC 27853), *Acinetobacter calcoaceticus* (clinical isolate), *Haemophilus influenzae* (ATCC 35036), and *Moraxella catarrhalis* (clinical isolate). Additionally, DNA extracted from human leukocytes was evaluated. All anaerobic bacterial strains and *S. intermedius* were cultured under anaerobic conditions on modified Gifu Anaerobic Medium agar (Nissui Pharmaceutical Co., Tokyo, Japan) plates. *S. intermedius* grows better under anaerobic conditions. Other aerobic bacterial strains were cultured in Luria broth (17), except for strains of *S. pneumoniae*, *K. pneumoniae*, *H. influenzae*, and *M. catarrhalis*, which were cultured in Trypticase soy broth (BBL Microbiology Systems). To calculate the detection limit for *B. fragilis* by the PCR method, serial dilutions of a suspension with *B. fragilis* were prepared as follows. Several colonies of *B. fragilis* cultured on modified Gifu Anaerobic Medium agar under anaerobic conditions for 2 days were suspended in sterile distilled water, and this suspension was diluted 10-fold with sterile distilled water. The number of bacterial cells was determined by inoculating 100 μl of each dilution onto modified Gifu Anaerobic Medium agar. Serial dilutions from 10⁴ CFU to 10⁻¹ CFU were used for the PCR described below.

**Patients and clinical specimens.** Thirty-nine specimens from 37 patients with infectious diseases were examined. (Three samples of bronchial aspirate obtained with a cannula from one patient with pneumonia were examined.) The clinical specimens used for detection of *B. fragilis* are classified in Table 1. Nine of 16 samples of bronchial aspirate obtained with a cannula, 3 of 3 samples of bronchial aspirate obtained with a PCB, 3 of 10 samples of percutaneous lung aspirate, and 2 of 2 samples of transtrachial aspirate were obtained from patients with pneumonia. Seven of 16 samples of bronchial aspirate obtained with a cannula and 7 of 10 samples of percutaneous lung aspirate were from patients with lung abscess. All six samples of pleural fluid were obtained from
glutamine synthetase gene of *B. fragilis* differs markedly from those of other prokaryotes and eukaryotes. Parts of the GS gene sequences that differ between *B. fragilis* and other prokaryotes and eukaryotes were chosen. BFR-1 (5'-ACTCTTT GTATCCCGACGATT-3') and BFR-2 (5'-GAGGTTGATGC CGTATCCCGATTAC-3') were used for the first PCR as the outer primers. BFR-1 and BFR-2 were located at nucleotides 484 to 504 and nucleotides 1045 to 1065, respectively, from the coding strand. BFR-3 (5'-GCTACCGAAGTTGCGCCAGCT-3') and BFR-4 (5'-GTGGTCATCCCGAGATTACA-3') were used for the second PCR as the inner primers. BFR-3 was located at nucleotides 580 to 600, and BFR-4 was located at nucleotides 901 to 921. BFR-5 (5'-AGGCCATTCAACTGGTTGG-3'), which was located at nucleotides 866 to 885, was used as a detection probe. These oligonucleotides were synthesized with a DNA synthesizer (model 380B; Applied Biosystems, Foster City, Calif.).

**DNA amplification.** For the first PCR, 10 µl of each extracted DNA was added to each reaction mixture, which contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.01% gelatin, 200 µM each dATP, dCTP, dGTP, and dTTP, 1.0 µM each of a set of primers (BFR-1 and BFR-2), 1.25 U of Taq DNA polymerase, and distilled water, in a final volume of 50 µl. Each reaction mixture was overlaid with a drop of mineral oil. After an initial denaturation step at 94°C for 10 min, 35 cycles of amplification were performed. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 63°C for 1.5 min, and extension at 72°C for 1 min. For the second PCR, 10 µl of each first PCR product was added to a freshly prepared reaction mixture containing BFR-3 and BFR-4 as primers. PCR amplification was performed under the conditions described above.

**Gel electrophoresis and Southern blot hybridization.** Ten microliters of each PCR product was electrophoresed with 2 µl of gel-loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll in water) through a 1.5% agarose gel at 100 V for 30 min in TBE buffer (45 mM Tris-HCl, 45 mM boric acid, 1 mM EDTA [pH 8.0]) containing ethidium bromide (0.5 µg/ml). A mixture of pH300PLK digested with HindIII and HaeIII and pH300.2PLK digested with HaeIII (Yakult Co., Tokyo, Japan) was used as a molecular weight marker. The gel was photographed under UV light.

For Southern blotting, the PCR products were transferred to a nylon membrane (Hybond-N+), using 0.4 N NaOH, and were fixed to the membrane by UV irradiation. The membrane was hybridized with the detection probe, BFR-5, by using an enhanced chemiluminescence 3'-oligolabeling and detection system (RPN 2131; Amersham International plc.). The specificity of the PCR reaction was confirmed by the presence or absence of the intended band.

**RESULTS**

**Specificity of the PCR.** Among 25 species (29 strains) of anaerobes, amplification of the 582-bp fragment by using BFR-1 and BFR-2 as primers was achieved only with the DNA isolated from *B. fragilis*. Twelve species of aerobes and human leukocytes were used for the study of specificity as well, but the 582-bp fragment was not obtained. The results for eight *Bacteroides* species (the former *B. fragilis* group) are shown in Fig. 1. The results for 5 strains of *B. fragilis*, 8 species of other anaerobes, and human leukocytes are shown in Fig. 2, and those for 12 species of aerobes are shown in Fig. 3. Weak bands very close to the 582-bp fragment of *B. fragilis* were noted for *B. caccae*, *B. merdae*, and *B. vulgatus* (Fig. 1A), but the 342-bp fragment was not obtained by the nested PCR method for those species (data not shown). Furthermore, a Southern blot hybridization assay confirmed that the bands

**FIG. 1.** Agarose gel electrophoresis (A) and Southern blot hybridization (B) of amplified DNA from *Bacteroides* sp. by using primers BFR-1 and BFR-2 and detection probe BFR-5. Lanes: 1, molecular size markers; 2, negative control (distilled water); 3, *B. fragilis*; 4, *B. caccae*; 5, *B. distasonis*; 6, *B. eggerthii*; 7, *B. merdae*; 8, *B. ovatus*; 9, *B. stercoris*; 10, *B. thetaiotaomicron*; 11, *B. uniformis*; 12, *B. vulgatus*.

**FIG. 2.** Agarose gel electrophoresis (A) and Southern blot hybridization (B) of amplified DNA from *Bacteroides* bacteria and human leukocytes by using primers BFR-1 and BFR-2 and detection probe BFR-5. Lanes: 1, negative control; 2, *B. fragilis* (type strain); 3, *B. thetaiotaomicron*; 4, *B. ureolyticus*; 5, *P. bivia*; 6, *P. intermedia*; 7, *P. oralis*; 8, *P. oris*; 9, *P. melanogenica*; 10, *Porphyromonas gingivalis*; 11, human leukocytes; 12 to 15, clinical isolates of *B. fragilis*.

were not specific to B. fragilis (Fig. 1B). Several bands of different molecular sizes were obtained for several species other than B. fragilis (Fig. 1A, 2A, and 3A), but a Southern blot hybridization assay subsequently confirmed that they were not specific to B. fragilis (Fig. 1B, 2B, and 3B). The results for the remaining eight anaerobic species also indicated that this method was specific for B. fragilis (data not shown).

**Sensitivity of the nested PCR method.** The sensitivity of the nested PCR method was evaluated by using serial dilution of bacterial cells and DNA of B. fragilis. In the bacterial cell study, 10³ CFU of B. fragilis was able to be detected by the first PCR, and one bacterial cell could be detected by the nested PCR (Fig. 4). Similarly, 1 ng of chromosomal DNA of B. fragilis was detectable by the first PCR, and 10 fg was detectable by the nested PCR (Fig. 5).

**Detection of B. fragilis from clinical specimens.** To evaluate the application of this PCR method to clinical specimens, 39 samples from 37 patients suspected of having anaerobic bacterial infections were examined. Four specimens that were positive by culture (two samples of bronchial aspirate from one patient with pneumonia, one sample of pleural fluid from a patient with pyothorax, and one sample of pus from a patient with a subcutaneous abscess from which B. fragilis was isolated) were all positive by both PCR and the hybridization assay with the PCR products (Table 1; Fig. 6). Two samples of bronchial aspirate were negative by culture but positive by PCR (Table 1; Fig. 6). One of these was the third specimen from a patient whose other two samples were positive by both culture and PCR. The first and second specimens had been cultured anaerobically immediately after collection, but the third specimen had been cultured several hours after collection. A Gram stain of this third specimen revealed some gram-negative bacilli, and only P. aeruginosa was isolated by culture. The other sample of bronchial aspirate that was negative by culture but positive by PCR was from another pneumonia patient. In this specimen, many gram-positive cocci were found by Gram stain, and Streptococcus mitis and aerobic gram-positive bacilli, which failed to be identified because of their inactive biochemical reactions, were isolated by culture. Neither of the DNAs extracted from these two bacterial strains was amplified by PCR with primers specific to B. fragilis. Clindamycin had been administered to both of these two patients before collection of the specimens. The remaining 33 specimens were negative by both culture and PCR. The gel electrophoresis and the hybridization assay of all the positive clinical specimens and a representative negative specimen are shown in Fig. 6. The negative specimen was a sample of bronchial aspirate from a patient with lung abscess, from which Streptococcus constellatus, F. nucleatum, and P. intermedia were isolated by culture.

**DISCUSSION**

Various methods to detect or identify B. fragilis and other Bacteroides spp. rapidly and accurately have been reported. By immunofluorescence, B. fragilis and members of the Bacteroides melaninogenicus group in clinical specimens can be detected directly and rapidly, even when they are not viable, but the method produces some false-positive results, and evaluation of unstable reactions is difficult (6, 21). DNA probes for Bacteroides spp. are more specific because these bacteria are differentiated at the genetic level. However, the limit of detection is 10³ bacteria, and therefore it is possible that false-negative results will be obtained. Also, culture is required to increase numbers of bacteria for direct detection from clinical specimens (10). The PCR method, which can amplify the specific gene fragment by as much as 10⁶-fold by using Taq DNA polymerase and specific primers for the target gene fragment in vitro, can detect and identify target organisms, even when they are not viable, with great specificity, sensitivity, and rapidity (16).

In this study, we developed specific primers for B. fragilis and used them to examine the PCR method. The method was specific for B. fragilis. No significant cross-reactivity with other anaerobes or aerobes was observed. In regard to sensitivity, we could detect as little as one bacterial cell of B. fragilis and 100 fg of DNA by the nested PCR method.

Regarding detection of B. fragilis in clinical specimens, all four of the culture-positive specimens were positive by PCR. Two specimens which were negative by culture were positive by PCR. The following are possible reasons for this finding: contamination of B. fragilis DNA, cross-reactivity with other
organisms, reaction to dead bacteria, or failure of the anaerobic culture process. The possibility of contamination is slight, since precautions to prevent it were taken during the process of sample preparation and DNA amplification and since target DNA amplified by this method was not observed in negative controls without template DNA, which were treated at the same time. Cross-reactivity with other organisms was ruled out by the negative results of PCR with DNA extracted from organisms isolated from these specimens. Because several hours had passed before the collected specimen was cultured anaerobically and because clindamycin had been administered to both patients before collection of these specimens, we regard these positive results as being caused by the reaction to DNA of dead B. fragilis. This ability to detect dead organisms is a great advantage of the PCR method for detection of anaerobes. Detection may be possible even when collection and transportation of the specimen are carried out improperly or when the specimen is obtained after the administration of antibiotics. Accordingly, the PCR method stands to become a very useful strategy for detection of anaerobes. However, there remains the problem of culture-negative but PCR-positive results. Hereafter, to establish the utility of this method, it will be necessary to examine a larger number of specimens, to continue comparing the results of PCR with those of culture, and to investigate the cause in cases of discrepancy. Various kinds of clinical specimens were used in this study, but comparison of results between the different specimens was not possible because of the small number of positive samples.

All specimens that showed positive results were highly purulent. DNA extracted from such specimens contains too much human DNA, which may obstruct PCR; nevertheless, we were able to detect target DNA from purulent specimens. On the other hand, since the volume of specimens obtained by bronchoscopy aspiration using a PCR or by percutaneous lung aspiration is too small, we had no positive results from such specimens in this study. From these data, we can conclude that as large a volume of the clinical specimen as possible must be taken, especially when the method of PCR or needle aspiration is used.

The PCR system investigated in this study was specific only for B. fragilis among Bacteroides spp. B. fragilis is isolated from clinical specimens most frequently, but other Bacteroides spp. are often also isolated, and they are as virulent and as resistant to many antimicrobial agents as B. fragilis. If a PCR system is developed, it may be more useful from the clinical point of view. As a further application, a PCR system for detection of the anaerobes P. melaninogenicus, F. nucleatum, and Peptostreptococcus spp., which are important in anaerobic infections of the lung and more difficult to isolate by culture, should be developed. Additionally, this system is expected to be extended to a wider variety of samples, such as blood and biopsied tissue. Thus, the PCR method shows much promise for the detection of anaerobes.

ACKNOWLEDGMENTS

We thank Kohei Hara for guidance and Kazue Ueno and Kunitomo Watanabe for kindly providing some anaerobic bacterial strains.

REFERENCES