**Association of Enterotoxigenic Bacteroides fragilis with Bacteremia**

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Polymerase chain reaction (PCR) assay was compared with cell culture assay performed with use of HT29/C1 (human colonic epithelial) cells for identifying strains of enterotoxin-producing *Bacteroides fragilis* (ETBF) isolated from extraintestinal specimens. A total of 188 unselected strains obtained over 2 years at a central clinical laboratory in Tokyo were tested. Overall, 35 strains (18.6%) were positive by cell culture and PCR assay, 152 strains were negative by both assays, and 1 strain was negative by cell culture assay but positive by the PCR assay; the same results were obtained in repeated assays. Among 64 strains from blood, 18 (28.1%) were ETBF, a rate that was significantly higher (*P* < .05) than the 17 ETBF (13.7%) among 124 strains from other sites. These results suggest that PCR assay is a simple and reliable tool for detecting ETBF and that enterotoxin may be a virulence factor in bacteremia caused by *B. fragilis*.

*Bacteroides fragilis* is an anaerobic microorganism that is frequently recovered from clinical specimens, particularly of abscesses. Some isolates of *B. fragilis* strains produce enterotoxin and may be responsible for diarrheal disease in humans and animals [1–3]. Recently, this enterotoxin was found to be a metalloprotease [4], suggesting that the enterotoxin may be a pathogenic factor in extraintestinal infections caused by *B. fragilis*.

Intestinal loop assays were used almost exclusively to detect and measure *B. fragilis* enterotoxin production until 1992, when the HT29/C1 cell line of human colonic epithelial cells was shown to be susceptible to *B. fragilis* enterotoxin [5]. Although an assay using HT29/C1 cells facilitated the detection of this toxin in research laboratories, the assay is subject to reader bias and variation, which can influence the sensitivity and specificity of the assay.

PCR assay is a valuable technique for detecting DNA sequences specific to organisms or for individual genes [6]. In general, PCR assays are more objective than cell culture assays for detecting toxins.

In this study we used a PCR assay to detect a segment of the *B. fragilis* enterotoxin gene previously reported by Moncrief et al. [4], and we compared the results with the results of cell culture assays performed with use of HT29/C1 cells. When the *B. fragilis* strains used in this study were restricted to isolates collected by a central clinical laboratory that serves all of Japan, the results of the study confirmed that enterotoxigenic *B. fragilis* (ETBF) is more prevalent in blood specimens than in other extraintestinal specimens.

**Materials and Methods**

*Bacteria tested.* A total of 188 unselected strains of *B. fragilis* were obtained from extraintestinal site specimens between 1987 and 1989 through the central clinical laboratory system in Tokyo, which has branches throughout Japan. Sixty-four strains were from blood, 20 were from ascitic sites, 7 were from bile, 2 were from pleural effusion, and 95 were from purulent material from a variety of other sources. Organisms were identified by the Rap ID ANA system (Innovative Diagnostic Systems, Atlanta) in conjunction with established methods [7, 8] at the Institute of Anaerobic Bacteriology (Gifu, Japan). The PCR assays were also utilized to identify *B. fragilis* by detection of the neuraminidase gene [9].

**Cell culture assay.** Cell culture assay to detect *B. fragilis* enterotoxin was performed with use of HT29/C1 cells as described previously [10], with the exception that the strains were cultured anaerobically in Fastidious Anaerobe Broth (Amersham, Amersham, England) for 24 hours. Morphological changes in the cell line were observed after 18–24 hours’ incubation. A sample was considered positive for *B. fragilis* enterotoxin if >25% of the cells were affected and if the changes were neutralized with antiserum to *B. fragilis* enterotoxin.

**DNA extraction and PCR methods.** A few colonies of organisms cultured on brucella HK medium (Kyokuto Seiyaku, Tokyo) were suspended in a solution consisting of 50 mM Tris-hydrochloride (pH, 8.0), 5 mM EDTA, and 50 mM NaCl, and then the solution was heated at 100°C for 2 minutes. Following centrifugation at 16,000g for 2 minutes, bacterial DNA for PCR was obtained in the supernatant.

The upstream primer GBF 101 and the downstream primer GBF 110 were used for the amplification of the *B. fragilis* enterotoxin gene. The sequences of oligonucleotide primers were as follows: GBF 101, 5′-GAGCCGAAGACGGTGTATTGT-3′ (corresponding to bases 1–20), and GBF 110, 5′-TCCCCACTGCGTCTAAAATTCCGAGC-3′ (bases 334–358; numbering is based upon the DNA sequences published by Moncrief et al. [4]). The expected PCR product for the primer set was 358 bp.
A total volume of 30 μL of reaction mixture contained (in final concentration) 10 mM Tris-hydrochloride (pH, 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.25 mM (each) deoxynucleoside triphosphate, 45 ng of each primer, 0.75 U of recombinant Taq DNA polymerase (Takara Biochemicals, Otsu, Japan), and 1 μL of bacterial sample DNA.

PCR was performed for 35 cycles. The two-step amplification cycle was performed at 95°C for 20 seconds and then at 68°C for 2 minutes. The final cycle was followed by an extension at 74°C for 5 minutes to ensure full extension of the product. A PC-700 thermal cycler (Astec, Tokyo) was used for amplification. Ten μL of amplification product was analyzed by gel electrophoresis at 125 V for 35 minutes on 5% polyacrylamide gels and visualized under an ultraviolet transilluminator following ethidium bromide staining.

Southern hybridization. The sequence of the oligonucleotide probe designated GBF 105 was 5'-CCAAAAGCAGAGTTATGAC-3' (bases 227–246) [4]. The probe was labeled with digoxigenin (DIG) by means of a DIG oligonucleotide 3’ end-labeling kit (Boehringer Mannheim Biochemical, Mannheim, Germany), according to the manufacturer’s directions. Southern hybridization was performed as described previously [9, 10].

In brief, after electrophoresis, PCR products were blotted onto a nylon membrane (Hybond-N, Amersham) with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The blots were then prehybridized for 30 minutes at 42°C in a hybridization solution containing 0.9 M NaCl, 6 mM EDTA, 20 mM Tris-hydrochloride (pH, 8.0), 0.1% SDS, and 0.25% nonfat milk. Next, hybridization was performed with a DIG-labeled probe at a concentration of 20 ng/mL in the hybridization solution for 60 minutes at 42°C. The membrane was washed twice (for 5 minutes each time) at room temperature with 2× SSC containing 0.1% SDS and then once for 15 minutes at 42°C with 0.1× SSC containing 0.1% SDS.

After a brief washing in washing buffer (0.3% Tween 20 plus buffer 1 [pH, 7.5], consisting of 0.1 M maleic acid and 0.15 M NaCl), the membrane was kept in blocking buffer (buffer 1 plus 1% casein) for 30 minutes at room temperature, incubated with alkaline phosphate–conjugated antibody to DIG for 30 minutes, and washed twice (for 15 minutes each time) with washing buffer. The membrane was rinsed briefly with detection buffer (0.1 M Tris-hydrochloride [pH, 9.5], 0.1 M NaCl, and 50 mM MgCl₂), incubated with a chemiluminescent substrate (CSPD; Boehringer Mannheim, Bedford, MA) for 1 hour, and then exposed to X-ray film.

Statistical analysis. The χ² test was performed for statistical analysis with use of StatView 4.0 (Abacus Concepts, Berkeley, CA).

Results

We initiated the study of PCR assay by testing a series of nine control strains. Representative results of PCR and Southern hybridization are shown in figure 1. The PCR products revealed a band of the predicted size. All six cell culture–positive strains were positive by PCR, and all three cell culture–negative strains were negative by PCR. These PCR results were consistent with the results of the Southern analysis.

We then tested 188 isolates of B. fragilis in parallel by cell culture and PCR assays. Overall, 35 strains (18.6%) were positive by both cell culture and PCR assays, 152 strains were negative by both assays, and 1 strain (isolated from blood) was negative by cell culture assay but positive by PCR assay; the same results were obtained after repeated assays.

By cell culture, 13 of 95 B. fragilis strains from pus (13.7%), 3 of 20 from ascitic sites (15.0%), 1 of 7 from bile (14.2%), 0 of 2 from pleural effusion, and 18 of 64 from blood (28.1%) were positive for enterotoxin. ETBF was isolated significantly more often from blood (18 of 64 specimens) than from other types of specimens (17 of 124; P < .05).

Discussion

In this study we compared the results of cell culture assays and those of PCR assays for detection of ETBF. We used Fastidious Anaerobe Broth instead of brain-heart infusion medium [2] or anaerobe broth MIC medium [11] since preliminary studies demonstrated that this broth supported the growth of B. fragilis better than did other media (data not shown). This choice of culture medium reduced some of the morphological changes that can obscure results with the cell line used.

The PCR results obtained with the 188 strains of B. fragilis in the study were consistent with those of the cell culture assay, indicating that PCR assay is an acceptable alternative to cell culture for detection of ETBF. One PCR-positive strain was negative repeatedly by cell culture, suggesting that either too little enterotoxin was produced by the strain to be detected by the cell culture assay used or else the toxin gene is simply not expressed or is truncated in some fashion.

The incidence of bacteraemia due to anaerobic bacteria has been reported to be decreasing at some hospitals in the United States [12, 13]. At one community hospital, however, the incidence of anaerobic bacteraemia was twofold higher than that reported in previous surveys [14]. These differences may depend on the types of hospitals studied, i.e., community vs. university/tertiary care hospitals [14]. Specimens used in this study were obtained from a number of community hospitals in Japan through a collaboration between a central clinical laboratory system and our institute from 1987 to 1989, allowing us to analyze a sufficient number of B. fragilis strains from blood; however, we were unable to investigate more recent isolates because only a few such isolates have been added to our collection since this collaboration ended.

B. fragilis is the most common anaerobic gram-negative organism isolated from blood [15]. So far, investigations have revealed that possible virulence factors of B. fragilis include capsules, fimbriae, outer membrane proteins, lipopolysaccha-
rides, enzymes, and metabolites [16–19]. Recent studies suggested that B. fragilis enterotoxin is a virulence factor of diarrhea. Our study demonstrated that ETBF was significantly associated with blood vs. other clinical specimens (P < .05), suggesting that enterotoxin may play an important pathogenic role in bacteremia caused by B. fragilis.

There are few reports in the literature regarding the correlation between enterotoxin production and bacteremia. Although Wachsmuth et al. reported that it seemed unlikely, at least in their study population, that toxin production was important in the pathogenesis of Escherichia coli bacteremia [20], Kasturi et al. demonstrated that 3 of 5 Salmonella typhimurium isolates from blood had enterotoxic activity, as demonstrated by fluid accumulation in a rabbit ileal-loop assay, and that only 2 of 15 isolates from feces were enterotoxigenic [21].

Safrin et al. reported that bacterial products, such as a cholerlike toxin and hemolysin, may play a role in the process of non-O:1 Vibrio cholerae bacteremia [22]. Myers and Weikel indicated no evidence of bacterial invasion or adherence to the intestinal mucosa of animals infected by ETBF [23]. However, the portal of entry for ETBF bacteremia may not always be the intestinal mucosa. It is very likely that most cases of ETBF bacteremia are caused by ETBF that has escaped from an abscess, wound, or other infected focus.

The way in which enterotoxin contributes to B. fragilis bacteremia remains unclear. Additional studies will be necessary to clarify the role of the toxin in the pathogenesis of bacteremia caused by B. fragilis. Evidence does exist that bacterial proteases play a role in the process of infection, including bacteremia [24, 25]. The fact that B. fragilis enterotoxin is a metalloprotease may support the hypothesis that this toxin acts to accelerate draining of B. fragilis into the bloodstream by some means, such as activation of the bradykinin cascade [25].

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References


