PCR Detection of the Bacteroides fragilis Enterotoxin Gene Relies on Robust Primer Design

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Colonial carriage of enterotoxigenic Bacteroides fragilis (ETBF) is reportedly more common in people presenting with colorectal cancer (CRC) than in healthy controls (1–3), fueling speculation that persistent carriage of these bacteria may “drive” colon carcinogenesis (4). Our aim was to use a bft amplification protocol to determine the prevalence of toxin-producing strains of B. fragilis in CRC patient stool samples.

Sixty-one patients histologically diagnosed with CRC consented to provide a stool sample prior to surgery. The study was approved by the Upper South A Regional Ethics Committee. Each stool was cultured anaerobically on Bacteroides bile esculin (BBE) agar (Fort Richard Laboratories, Auckland, New Zealand). After 48 h of incubation at 37°C, all colonies were swept off the plates into 500 μl of sterile water and heated for 10 min at 99°C. Each sample was screened for evidence of the bft gene using Pantosti (5) and Odamaki (6) primer sets. The PCR mixtures for the Pantosti toxin primers consisted of a 10-μl volume containing 5 pmol of each primer, 200 nM deoxynucleoside triphosphates (dNTPs), 2 mM MgCl2, 1× enzyme buffer, 0.5 U HotFire polymerase (Solis Biodyne, Tartu, Estonia), and 0.5 μl DNA template. The PCR mixture for amplification of the toxin gene by Odamaki primers differed by using 1.5 mM MgCl2 and by the addition of 1 mM of S solution (Solis Biodyne, Tartu, Estonia). Amplification consisted of a 15-min incubation at 95°C followed by 35 cycles of 30 s of denaturation at 95°C, 30 s of annealing (52°C and 66°C, respectively), and 30 s of extension at 72°C, followed by a final extension step of 2 min at 72°C. Positive and no-template controls were included in each experiment. Products were included on a 1.5% agarose gel, and fragments were visualized using SYBR Safe dye (Invitrogen, Carlsbad, CA, USA).

Results showed that the Pantosti primers amplified only 5 ETBF samples while the Odamaki primers amplified 10. Only four samples amplified by Odamaki primers were recognized by the Pantosti primers, while one sample amplified by the Pantosti primers was not recognized by the Odamaki primers (Table 1). ETBF-positive samples were further analyzed by subtype-specific PCR (6). Notably, the Pantosti primers gave no discernible product other than BFT-1, whereas samples that yielded positive bft fragments with Odamaki primers only were identified as containing BFT-2 and -3 subtypes. This finding was confirmed using both primer sets to amplify DNA extracted from three ETBF reference strains (strains VPI 13784 [BFT-1] [7], 86-5443-2-2 [BFT-2] [8], and Korea 570 [BFT-3] [9], generously supplied by Cynthia Sears). The Odamaki primer set amplified all three subtypes well, whereas the Pantosti primers amplified less efficiently from BFT-2 and -3 subtypes. This finding was confirmed using both primer sets to amplify DNA extracted from three ETBF reference strains (strains VPI 13784 [BFT-1] [7], 86-5443-2-2 [BFT-2] [8], and Korea 570 [BFT-3] [9], generously supplied by Cynthia Sears). The Odamaki primer set amplified all three subtypes well, whereas the Pantosti primers amplified less efficiently from BFT-2 and -3 very poorly from BFT-3 (Fig. 1). Collectively, these results suggest that Pantosti primers may miss some BFT-2- and BFT-3-containing samples.

The Pantosti primer set, still in common use (3, 10), was designed against the sequence published by Moncrief and colleagues.

<table>
<thead>
<tr>
<th>Study and subtype</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Forward primer (5′–3′)</td>
</tr>
<tr>
<td>Odamaki et al. (6)</td>
<td></td>
</tr>
<tr>
<td>BFT-1</td>
<td>GGATACATCACTACGCGGTGTAG</td>
</tr>
<tr>
<td>BFT-2</td>
<td></td>
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<tr>
<td>BFT-3</td>
<td></td>
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<tr>
<td>Pantosti et al. (5)</td>
<td></td>
</tr>
<tr>
<td>BFT-1</td>
<td>GACGCGTGTATACTGAGATTTTGTGTTAG</td>
</tr>
</tbody>
</table>
| BFT-2                   | A---------A---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------C---------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leagues, who used a predicted DNA sequence from the amino acid sequence of the toxin to generate a primer at the start of their sequence (11), an approach that would generate multiple possible variations due to degeneracy within the protein. The Pantosti forward primer uses the final 19 bases of the Moncrief primer, plus 8 bases from the sequenced \( bft \). Therefore, while the Pantosti forward primer contains mismatches, the 3' end is a good match for \( bft \). However, the reverse primer, although a good match for the published sequence (Fig 1), contains multiple mismatches for \( bft \) and particularly for \( BFT-3 \) (Table 1), and this may explain the observed bias toward detection of the \( BFT-1 \) subtype when these primers are used.

In contrast, the primers generated by Odamaki et al. have 100% identity to published sequences of all three \( BFT \) subtypes (Table 1), similar to primer sets used by Kato et al. (12) and Franco et al. (13), despite different accession numbers for the sequences from which they derived primers. However, even when two primer sets with 100% identity to published \( bft \) sequences are used, differences in sensitivity may still occur, and the use of two primer sets may increase identification of ETBF in clinical samples (1).

We did not use any other of the published \( bft \) primer sequences to screen our study population. However, we note that the primers used by Toprak and colleagues (2) are also designed against the Moncrief sequence and, accordingly, are markedly different from the published \( bft \) sequences (Table 1). This study reports a high carriage rate for ETBF among CRC patients, and with no reference to sequencing of PCR products, the possibility that these primers are associated with a high rate of false negatives cannot be excluded.

It is, however, important to note that the \( bft \) sequence originally submitted by Moncrief and colleagues in 1995 with the erroneous 27-base primer sequence still exists in the GenBank database (S75941.1) and that a BLAST search of any of these forward primers will produce a hit with 100% identity, giving the user false confidence that the primers are specific.

In conclusion, our study highlights the care that must be taken when designing PCR-based studies to measure ETBF carriage rates to ensure that primers used are designed against robust \( bft \) sequences that have strong identity to all three \( bft \) subtypes. If not, these studies may inadvertently under- or overestimate the incidence of toxin-producing strains of \( B. fragilis \) in their samples.

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**REFERENCES**


