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MAJOR ARTICLE

The *Bacteroides fragilis* Toxin Gene Is Prevalent in the Colon Mucosa of Colorectal Cancer Patients

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Background. Enterotoxigenic *Bacteroides fragilis* (ETBF) produces the *Bacteroides fragilis* toxin, which has been associated with acute diarrheal disease, inflammatory bowel disease, and colorectal cancer (CRC). ETBF induces colon carcinogenesis in experimental models. Previous human studies have demonstrated frequent asymptomatic fecal colonization with ETBF, but no study has investigated mucosal colonization that is expected to impact colon carcinogenesis.

Methods. We compared the presence of the *bft* gene in mucosal samples from colorectal neoplasia patients (cases, n = 49) to a control group undergoing outpatient colonoscopy for CRC screening or diagnostic workup (controls, n = 49). Single bacterial colonies isolated anaerobically from mucosal colon tissue were tested for the *bft* gene with touch-down polymerase chain reaction.

Results. The mucosa of cases was significantly more often *bft*-positive on left (85.7%) and right (91.7%) tumor and/or paired normal tissues compared with left and right control biopsies (53.1%; $P = .033$ and 55.5%; $P = .04$, respectively). Detection of *bft* was concordant in most paired mucosal samples from individual cases or controls (75% cases; 67% controls). There was a trend toward increased *bft* positivity in mucosa from late- vs early-stage CRC patients (100% vs 72.7%, respectively; $P = .093$). In contrast to ETBF diarrheal disease where *bft-1* detection dominates, *bft-2* was the most frequent toxin isotype identified in both cases and controls, whereas multiple *bft* isotypes were detected more frequently in cases ($P \leq .02$).

Conclusions. The *bft* gene is associated with colorectal neoplasia, especially in late-stage CRC. Our results suggest that mucosal *bft* exposure is common and may be a risk factor for developing CRC.

Keywords. enterotoxigenic *Bacteroides fragilis*; mucosal microbiota; colorectal cancer; *Bacteroides fragilis* toxin.

The anaerobe *Bacteroides fragilis* is a common colonic symbiote with an affinity for mucosal colonization but is also known to comprise only a small proportion of the fecal microbiota (approximately 0.5%–1%) [1, 2]. There are 2 molecular subtypes, nontoxigenic *B. fragilis* (NTBF) and enterotoxigenic *B. fragilis* (ETBF). Nearly 30 years ago, ETBF was implicated as causing diarrheal illnesses affecting livestock [3] and humans [4]. ETBF is now established as a cause of diarrheal disease in all age groups globally, with most reports focusing on young children [5]. Limited data also support an association of ETBF with active inflammatory bowel disease (IBD) [6, 7] and colorectal cancer (CRC) [8, 9]. Similar to other enteric pathogens, asymptomatic ETBF colonization is detected in children and adults with carriage rates as high as 40% in fecal samples from healthy adults [10].

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ETBF pathogenicity is due to the *B. fragilis* toxin (BFT), a 20 kDa zinc-dependent metalloprotease toxin with 3 isotypes (BFT-1, BFT-2, and BFT-3) [11]. Sequence analysis indicates that the *bft* gene is unique and, since cloned in 1995 [12], only identified in *B. fragilis*. In vitro BFT binds to a specific colonic epithelial receptor activating Wnt and NF-κB signaling pathways with increased cell proliferation, epithelial release of proinflammatory mediators, and induction of DNA damage [5, 13–16]. In vivo ETBF, but not NTBF, induces BFT-dependent acute and chronic colitis in C57BL/6 mice [11, 17]. In multiple intestinal neoplasia ($Min^{Apc+/-}$) mice, a model for human CRC, ETBF promotes interleukin 17 (IL-17)–dependent carcinogenesis [8]. These data suggest that ETBF is a candidate etiologic agent in human sporadic CRC.

To further address the role of ETBF in the pathogenesis of human CRC, characterizing mucosal exposure to BFT is critical because long-term mucosal exposure is hypothesized to contribute to colon neoplastic transformation. Herein, we present novel data on the detection of the *bft* gene, the critical virulence determinant of ETBF, in mucosal samples from colorectal neoplasia patients (cases) compared with individuals undergoing outpatient colonoscopy (controls).

MATERIALS AND METHODS

Patient Population

Adult patients with colorectal neoplasia (cases; 43 = CRC, 6 = adenomas) undergoing primary colorectal surgical resections at Johns Hopkins Hospital (JHH) were studied between May 2010 and September 2012. Only tissue not needed for pathologic diagnosis was collected. Individuals undergoing outpatient colonoscopy (controls) at JHH between August 2011 and February 2013 for routine CRC screening or a diagnostic workup (eg, for anemia) were also studied.

Exclusion Criteria

Cases who received preoperative radiation and/or chemotherapy or with a history of CRC or IBD were excluded [18–20]. Similarly, controls with a history of CRC, IBD, or chemotherapy within 2 years of their procedure were excluded.

Antibiotic Exposure

A subset of cases received preoperative mechanical bowel preparation (MBP) without or with oral antibiotics, most often neomycin and erythromycin (MBP-No Abx vs MBP-Abx) (Table 1). Preoperative intravenous antibiotics were administered to all cases (cefotetan or clindamycin/gentamicin) within 1 hour of skin incision. In January 2012, JHH protocols changed to comply with newly emerging surgical infection prophylaxis guidelines [21] advocating MBP-Abx prior to all colorectal surgical procedures for surgical site infection prophylaxis.

Table 1. Characteristics of Cases and Controls

Characteristics	All Cases (n = 49)	No Abx Cases (n = 26)	Controls (n = 49)
Age, y, median (IQR)[a]	62 (52–76)	64 (52.2–75.2)	62 (49–66)
Male sex[b]	22 (44.9)	11 (42.3)	20 (40.8)
Race[c]			
White	38 (77.6)	20 (76.9)	18 (36.7)
African American	8 (16.3)	4 (15.4)	26 (53.1)
Other	3 (6.1)	2 (7.7)	5 (10.2)
Bowel preparation			
No prep	20 (41)	20 (76.9)	0
MBP-No Abx	6 (12)	6 (26.1)	49 (100)
MBP-Abx	23 (47)	0 (0)	0
Indication for procedure			
Screening	NA	NA	30 (61.2)
Diagnostic workup	NA	NA	19 (38.8)
Colorectal tumor	49 (100)	26 (100)	NA
Histologic diagnosis			
Total tumors	51	28	NA
Tubular adenoma	5 (10.2)	3 (10.7)	11 (22.4)[d]
Tubulovillous adenoma	3 (5.9)[e]	2 (7.1)[e]	NA
Adenocarcinoma	43 (84.3)[f]	23 (82.1)[f]	NA
Stage I[g]	7 (16.3)	4 (17.4)	NA
Stage II[g]	12 (27.9)	7 (30.4)	NA
Stage III[g]	11 (25.6)	7 (30.4)	NA
Stage IV[g]	13 (30.2)	5 (21.7)	NA
Tumor size, cm, median (IQR)	4.5 (1.8)	4.4 (1.9)	NA

Data are presented as No. (%) unless otherwise specified.

Abbreviations: Abx, antibiotics; IQR, interquartile range (defined as quartile 3 - quartile 1); MBP-Abx, mechanical bowel preparation with oral antibiotics; MBP-No Abx, mechanical bowel preparation without oral antibiotics; NA, not applicable.

[a] *t* test, $P = .248$ (all cases) and $P = .367$ (No Abx cases), compared to controls.

[b] Fisher exact test, $P = .838$ (all cases) and $P = 1.00$ (No Abx cases), compared to controls.

[c] χ^2 test of independence, $P = .002$ (all cases) and $P < .001$ (No Abx cases), compared to controls.

[d] Eleven of the 49 controls had tubular adenomas removed during colonoscopy.

[e] One patient with 2 tubulovillous adenomas.

[f] One patient with both an adenocarcinoma and tubulovillous adenoma.

[g] The age distribution was similar between stage I/II and stage III/IV cases ($\chi 2$ test, $P = .763$).

History of antibiotic use within 12 months preceding colonoscopy was assessed by questionnaire. Oral antibiotics were not part of colonoscopy MBP.

Study Approval

This study was approved by the JHH Institutional Review Board. All samples were obtained in accordance with the Health Insurance Portability and Accountability Act.

Sample Collection

Mucosal tissue punches (4, 5 or 8 mm) from paired tumor and grossly normal tissue (Supplementary Figure 1) were harvested from the surgical specimens. Tissue pairs proximal to or from the hepatic flexure were defined as right colon while specimens distal to the hepatic flexure were defined as left colon. Colonoscopy biopsies were obtained from the right (cecum or ascending) and/or left (descending or sigmoid) colon using 2.8-mm disposable biopsy forceps (Boston Scientific Corporation). Surgical specimens were exposed to air for up to 45 minutes prior to tissue collection; colonoscopy biopsies were exposed to air ≤30 seconds.

Tissue Processing

Sample pairs from cases or controls were processed by 1 or both approaches as follows:

Broth Single Colony Method

Tissue samples were placed in peptone yeast glucose bile broth and then in an anaerobic chamber (Anaerobe Systems) at 37°C. Turbid broth (25 µL) was then inoculated on *Brucella* blood agar (BRU) (nonselective medium; Anaerobe Systems) and *Bacteroides* bile esculin agar (BBE) (*Bacteroides* selective; Becton Dickinson) to obtain single colonies (approximately 48–72 hours). BRU colonies were reisolated on BBE to select *Bacteroides* species. From each sample, 8–16 BBE isolates were expanded on tryptic soy agar with 5% sheep blood (TSA) (Anaerobe Systems) and tested for the *bft* gene.

Direct Single Colony Method

Mucosal tissue samples collected in anaerobic transport medium (ATM) (Anaerobe Systems) were washed twice with 0.016% DL-dithiothreitol in saline prior to pestle homogenization in sterile phosphate-buffered saline in an anaerobic chamber. Homogenized tissue dilutions (10^0–10^{-6}, 25 µL each) were inoculated on BRU and BBE agar and 8–16 colonies per sample were tested for the *bft* gene and expanded on TSA as above. Colony-forming unit (CFU) counts were obtained from BBE agar.

Unless otherwise stated, the data from the 2 processing methods were combined for presentation because the results did not differ (78.3% concordant; $P = 1.00$ [McNemar test]; data not shown). On average, a total of 32 colonies per patient were analyzed for the *bft* gene for both cases and controls (see also "Results" section).

bft Polymerase Chain Reaction Analysis

Colonies from TSA plates were boiled and supernatant was used as DNA template for touch-down polymerase chain reaction (PCR) amplification evaluating 368-bp and/or 281-bp regions of the *bft* gene. PCR reactions used Platinum PCR SuperMix (Life Technologies Corporation) and 1 µM of forward and reverse primers according to protocol on a thermocycler (Applied Biosystems) (Supplementary Table 1) [22, 23]. PCR products were evaluated on 1.5% low-melting agarose gels and stained with ethidium bromide.

bft Isotype Identification

PCR products from *bft*-positive bacterial colonies were purified using the QIAquick PCR Purification Kit protocol (Qiagen) and sequenced (Genewiz, Inc) to determine *bft* isotypes. Sequences were screened with BLASTN against the National Center for Biotechnology Information nucleotide database, and isotype was verified at 99% identity and coverage.

Statistical Analysis

Patients were considered *bft*-positive if at least 1 bacterial colony from any tissue sample was *bft*-positive. Patient characteristics were compared using unpaired *t* test, Fisher exact test, or χ^2 test as appropriate. The prevalence of *bft* between cases and controls and among tissue groups was compared using, as appropriate, McNemar, χ^2, or Fisher exact test. CFU counts between cases and controls were analyzed using the Mann–Whitney *U* test, and *bft* isotypes between a subset of cases and controls with χ^2 test. All statistical tests were performed with GraphPad Prism 5.0 and GraphPad InStat 3.05 and $P < .05$ defined as significant.

RESULTS

Patient Characteristics

A total of 49 cases with 8 adenomas and 43 adenocarcinomas (1 patient, 2 adenomas; 1 patient, CRC and adenoma) and 49 controls were studied (Table 1, Supplementary Table 2). Median age (62 years) and sex distribution were similar between cases and controls. Cases were significantly more often white, compared with controls who were more often African American ($P = .002$). Of the 51 tumor samples analyzed from cases, 47.1% (24/51) were from the left colon. Most controls (n = 30 [61.2%]) were undergoing CRC screening, with 91.8% having both right and left colon biopsies obtained (Supplementary Table 2).

Among cases, 23 patients (46.9%) had MBP-Abx within 24 hours prior to their operation, 20 (41%) had no MBP, and 6 (12%) had MBP-No Abx (Table 1). Among controls, 12 (24.5%) reported a history of antibiotic exposure within 12 months prior to their procedure, with 58.3% (7/12) reporting antibiotics within 3 months of colonoscopy. Only 2 patients reported taking antibiotics at the time of colonoscopy.

bft Is More Prevalent in Mucosal Samples From Cases Than Controls

Analysis of single bacterial colonies was pivotal in establishing the role of enterotoxigenic *Escherichia coli* as an etiology of diarrheal disease [24]. Thus, we analyzed individual *Bacteroides* colonies from BBE plates for the *bft* gene to detect *bft* positivity. Because antibiotic exposure could confound *bft* detection, *bft*

Table 2. Case and Control *bft* Status by Single *Bacteroides* Colony Analysis

	Cases[a]				Controls				
Patients	Total	No Abx[b]	MBP-Abx	*P* Value[c]	Total	No Abx	Abx	*P* Value[c]	*P* Value[d]
Total									
No.	49	26	23		49	37	12		
No. *bft*$^+$ (%)	33 (67.3)	23 (88.5)	10 (43.5)	.002	33 (67.3)	24 (64.9)	9 (75.0)	.726	.054
Left-sided									
No.	24	14	10		49	37	12		
No. *bft*$^+$ (%)	18 (75.0)	12 (85.7)	6 (60.0)	.192	26 (53.1)	21 (56.7)	5 (41.7)	.508	.033
Right-sided									
No.	27	12	15		45	34	11		
No. *bft*$^+$ (%)	16 (59.3)	11 (91.7)	5 (33.3)	.005	25 (55.5)	17 (50.0)	8 (72.7)	.297	.040
P value[e]	.372	1.00	.414		.838	.638	.214		

Case or control *bft* positivity is based on combined direct and broth single colonies from BBE plates (Supplementary Materials and Methods); all *P* values were calculated with Fisher exact test. Note that left-sided and right-sided cases does not equal total cases; 2 patients had both a left and right sided tumor and are represented in both categories (see Table 1).

Abbreviations: Abx, antibiotics; BBE, *Bacteroides* bile esculin agar; MBP-Abx, mechanical bowel preparation with oral antibiotics; MBP-No Abx, mechanical bowel preparation without oral antibiotics.

[a] Case specimens include analysis of patients with adenomas (n = 6) or carcinomas (n = 43) and their paired normal tissues (Supplementary Methods). *P* values were calculated using Fisher exact test. For controls, exposure to antibiotics was determined by questionnaire (Supplementary Materials and Methods).

[b] No Abx cases includes MBP-No Abx and No Prep (see Table 1)

[c] MBP-Abx vs No Abx groups for cases; Abx vs No Abx groups for controls.

[d] Total controls vs No Abx cases.

[e] Left-sided vs right-sided.

positivity between cases with and without MBP-Abx was compared. Our initial analysis revealed a marked effect of MBP-Abx on bacterial recovery. Among MBP-Abx cases (n = 23), 70% of either tumor or paired normal colon samples did not culture any bacteria compared with no samples from cases without antibiotics (n = 26; Table 1) and, similarly, median *Bacteroides* species. CFUs were significantly lower in samples from MBP-Abx cases (8.0 CFU/sample; interquartile range [IQR], 2–6.6×10^1) vs cases without antibiotics (5.0×10^2 CFU/sample; IQR, 3×10^1–5×10^3) (Mann–Whitney *U* test, $P < .001$; Supplementary Figure 2). Consistent with these data, the number of *bft*-positive cases was significantly lower in those who received MBP-Abx (43.5% vs 88.5%; $P = .002$). In contrast, *bft* positivity was similar in controls regardless of reported antibiotic exposure (Table 2). The 2 controls who reported current antibiotic use were both *bft*-positive. Three (6.1%) controls did not have any bacterial growth; none reported antibiotic exposure.

Because antibiotic exposure did not modify *bft* results in controls, we next compared all MBP-No Abx cases to all controls. This analysis suggested that cases were more often *bft*-positive than controls (88.5% vs 67.3%, respectively; $P = .054$; Figure 1*A*; Table 2). Importantly, *bft* positivity did not differ by race in cases (white, 85.0% vs African-American, 100.0%; $P = 1.00$) and controls (white, 60.0% vs African-American, 73.1%, $P = .492$). Further stratification by left vs right colon tumors revealed a significant association of *bft* detection in cases compared to corresponding left vs right control biopsies ($P = .033$ and $P = .040$, respectively; Figure 1*A*, Table 2). All 3 cases with surgically removed tubulovillous adenomas were *bft*-positive whereas among controls, *bft*-positive status did not differ between individuals with (n = 11 [54.5%]) or without (n = 38 [71.1%]) small tubular adenomas removed at colonoscopy (Fisher exact test, $P = .466$; Supplementary Table 2*B*).

A potential limitation is the number of colonies that were examined in cases and controls. A median of 32.0 (IQR, 24.7–40.5) colonies from cases and 32 (IQR, 27.0–48.0) from controls were examined (Mann–Whitney *U* test, $P = .857$; Supplementary Figure 3*A*). However, when the single colonies evaluated per tissue sample were corrected for tissue size (mm^2), the number of colonies tested in controls was approximately 3.5 times greater than for cases (Mann–Whitney *U* test, $P < .001$; Supplementary Figure 3*B*), suggesting a possible underrepresentation of the results in cases. Despite this potential bias, a significantly higher *bft* frequency was detected in MBP-No Abx cases compared with controls in both the right and left colon (Figure 1*A*).

The Majority of Paired Tissue Samples Are Both *bft*-Positive

Next, we analyzed whether *bft* detection differed between tumor and normal tissue pairs from MBP-No Abx cases or between right and left colonoscopy biopsy pairs within a single control (Supplementary Figure 4). From cases, 24 tumor/normal tissue

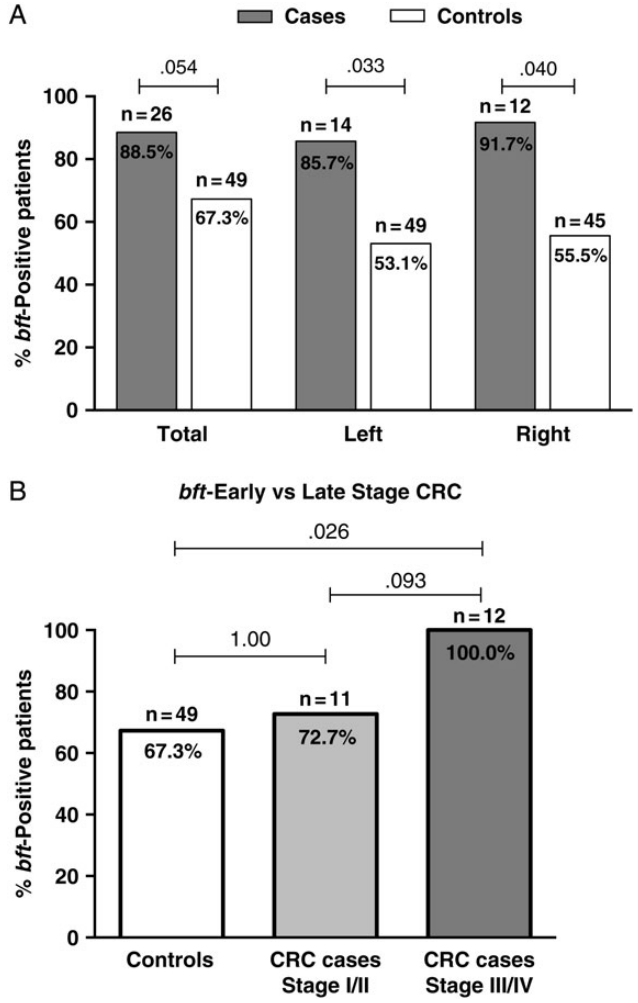

Figure 1. Case and control *bft* status. *A*, Overall, cases (n = 26; no antibiotics) were more often *bft*-positive than controls (n = 49; $P = .054$). *bft* positivity was present significantly more often in both left- and right-sided colorectal neoplasia cases compared with left and right colon biopsies from controls (Fisher exact test, $P = .033$ and $P = .040$, respectively). *B*, Patients with colorectal cancer (CRC) (n = 23; no antibiotics) were stratified by tumor stage (early, stage I/II; and late, stage III/IV). All 12 late-stage CRC patients (100%) were *bft*-positive compared with 67.3% of all controls and 71.1% of controls without adenomas (Fisher exact test, $P = .026$ and $P = .046$, respectively). Cases with adenomas were excluded from this analysis.

pairs were analyzed and, from controls, 35 left/right biopsy pairs. Among the 24 tumor/normal pairs, 13 (54.2%) tissue pairs were both *bft*-positive. In 7 (29.2%) pairs, only 1 sample (tumor or normal) was *bft*-positive, whereas in 4 pairs (16.7%) *bft* was not detected in either sample. Among 35 left/right biopsy pairs from controls, 10 (28.6%) biopsy pairs were both *bft*-positive; in 11 (31.4%) pairs, only 1 biopsy (left or right) was *bft*-positive; and in 14 (40.0%) pairs, both were *bft*-negative. The frequency of *bft* detection on tumors vs normal tissues in cases or right vs left colon biopsies in controls did not differ (McNemar test, tumor vs normal, $P = 1.00$; right vs left biopsies, $P = 1.00$). An analysis of the median percentage of *bft*-positive colonies revealed a nonsignificant trend ($P = .477$), with the highest number of *bft*-positive colonies detected on tumors and lowest on control biopsies (tumor, 18.8%; paired normal, 12.5%; biopsies 6.3%) (Supplementary Figure 5). Thus, individual patient tumor/normal pairs were concordant for *bft* status in 75% of cases and 67% of controls. Altogether, these data suggest that mucosal *bft* presence is not limited to tumors but spans a larger portion of the colonic mucosa.

Late-Stage CRC Patients Have Higher *bft* Detection Than Early-Stage CRC Patients

We examined whether *bft* prevalence differed by cancer stage in patients with MBP-No Abx CRC (early-stage [stage I/II] vs late-stage [stage III/IV]; Table 1). All late-stage CRC patients (100%) were *bft*-positive compared with 72.7% of early-stage CRC patients ($P = .093$; Figure 1*B*). When compared to the overall *bft*-positive rate in controls (67.3%) or in controls without tubular adenomas detected on colonoscopy (n = 38 [71% *bft*$^+$]), *bft* detection was significantly higher among late-stage CRC patients ($P = .026$ and $P = .046$, respectively).

Multiple *bft* Isotypes Are Present in Mucosal Samples of Cases

From a subset of patients (28 cases [n = 24 CRC; n = 4 adenomas] and 32 controls), single-colony *bft* PCR-products were purified and sequenced to identify the *bft* isotype. In total, 103 and 122 *bft*-positive colonies were sequenced from cases and controls, respectively. Overall, *bft-2* was the most commonly identified isotype on the colon mucosa in both cases (41.2%) and controls (57.6%) ($P = .226$; Table 3). Multiple *bft* isotypes, most often *bft-1* and *bft-2*, were detected significantly more often in cases (67.8%) than in controls (34.4%) ($P = .019$; Table 3).

DISCUSSION

Our key finding is that there is a significant association of *bft* detection in left- and right-sided colon mucosal samples from cases compared with controls with 100% of late-stage CRC identified as *bft*-positive. This supports prior work where *bft* detection in stools was significantly higher in hospitalized CRC patients than outpatient controls [9]. Additionally, *bft* mucosal detection was common in our controls and higher than prior results based on fecal analyses of adults (40%) [10]. The colon mucosal microbial community is either unique or a subset of that detected in feces [25, 26] with Bacteroidetes and, specifically *B. fragilis*, reported as more abundant in mucosal than luminal samples in CRC patients [27, 28]. In vivo experimental studies show that ETBF is highly carcinogenic and in vitro studies demonstrate potential mechanisms for colon epithelial cell

Table 3. Determination of *bft* Isotype by Sequence Analysis of Polymerase Chain Reaction Amplicons

bft Isotype Status	Cases[a]	Controls
Patients	28	32
Single isotype	9 (32.1)	21 (65.6)
bft-1	3 (10.7)	7 (21.9)
bft-2	6 (21.4)	13 (40.6)
bft-3	0 (0.0)	1 (3.1)
Mutiple isotypes[b]	19 (67.9)	11 (34.4)
bft-1 and *-2*	16 (57.1)	9 (28.1)
bft-1 and *-3*	1 (3.6)	0 (0.0)
bft-2 and *-3*	1 (3.6)	1 (3.1)
bft-1, -2 and *-3*	1 (3.6)	1 (3.1)
Overall isotype frequency[c]		
bft-1	21 (43.8)	17 (38.6)
bft-2	24 (50.0)	24 (54.5)
bft-3	3 (6.3)	3 (6.8)
Single colonies[d]	103	122
bft-1	38 (24.8)	32 (21.2)
bft-2	63 (41.2)	87 (57.6)
bft-3	2 (1.3)	3 (2.0)

Data are presented as No. (%).

Abbreviation: MBP-Abx, mechanical bowel preparation with oral antibiotics.

[a] Includes 7 MBP-Abx cases that grew bacteria.

[b] Cases had multiple *bft* isotypes detected compared with controls (Fisher exact test, $P = .019$).

[c] *bft*-2 was the most common isotype and did not differ between cases and controls (χ^2 test of independence, $P = .884$). The numbers represent patients with single and multiple isotypes.

[d] Total single colonies isolated from cases or controls; the distribution of isotypes was not different (χ^2 test of independence, $P = .226$). Numbers represent total isolated colonies that are *bft*$^+$.

oncogenic transformation [8, 11, 13, 16]. The prevalent mucosal detection of *bft* suggests that ETBF may be one member of the microbiota contributing to colon carcinogenesis.

Unexpectedly and in contrast to work using fecal samples, where *bft-1* detection is most common [5], our results show *bft*-2 as the most common mucosal isotype. Furthermore, cases significantly more often had multiple *bft* isotypes compared with controls, an observation not reported before in humans. In previous work, approximately 65% of fecal ETBF strains harbored *bft*-1 compared with approximately 25% *bft-2* and approximately 10% *bft-3* [5, 9, 22, 29, 30]. In 1 prior study of CRC patients, subtyping fecal ETBF also revealed predominant *bft*-1 (87.1%), not significantly different from controls (*bft*-1, 87.5%) [9]. Notably, BFT-2 has greater potency and biologic activity in vitro and in vivo compared to BFT-1 and, in preliminary data, is more carcinogenic in $Min^{Apc+/-}$ mice (Wu and Sears, unpublished data) [5, 31]. Collectively, this suggests that *bft-2*-expressing stains exhibit enhanced mucosal adherence and carcinogenic potential compared to *bft*-1 strains, supporting a role for *bft* in the initiation and/or progression of human CRC.

Our study differs in both patient characteristics and microbiology methods compared to previous work. Zitomersky et al detected fecal ETBF in 40% (6/15) of healthy adults (mean age, 42 years) whom had on average 29 Bacteroidales analyzed per stool sample; *bft* was detected in 56.7% (101/178) of all *B. fragilis* isolates [10]. Subsequent work by this group, in contrast, identified mucosal ETBF in only 5% of IBD patients (mean age, 15–16 years; n = 63) and controls (mean age, 14 years; n = 31) and approximately 6% (6/104) of all isolates [32]. We studied an older population (median age, 62 years) and analyzed multiple mucosal samples per patient (mean, 28 colonies/patient). One critical methodology difference may be our use of ATM prior to homogenization and plating of samples in an anaerobic hood. This markedly enhanced *bft* detection (50% *bft* positivity without ATM vs 89% *bft* positivity with ATM, $P = .024$; manuscript in preparation). Overall, mucosal *bft* detection using our single-colony methodology was notably higher (67.3% of controls and 17.3% of all isolates examined).

In our study, *bft* was detected in the majority of surgically resected tumors and was uniform in late-stage CRCs, possibly due to enhanced anaerobiosis on larger tumors. This contrasts with *Helicobacter pylori* gastric colonization that diminishes in gastric cancer compared with earlier disease, as metaplastic tissue appears to be less hospitable for *H. pylori* [33–35]. Recent data suggest *B. fragilis* preferentially colonizes colonic epithelial crypts and, thus, may exhibit more stable colonization in CRC through evasion of host immune responses [36]. Crypt accumulation of ETBF strains over time expressing different *bft* isotypes may enhance carcinogenesis. ETBF induces rapid onset of chronic IL-17–dependent colitis and tumor formation in $Min^{Apc+/-}$ mice with foci of persistent Stat3 activation [8, 17] and reactive oxygen species with DNA damage, potent mediators of oncogenesis [16, 37]. We postulate that *bft* exposure in the human colon may induce chronic, perhaps focal, mucosal inflammation yielding sites prone to DNA mutagenesis and carcinogenesis.

There are several important considerations for interpreting these study results. First, MBP-Abx prior to colorectal surgery significantly reduced *bft* recovery from surgical specimens and limited, over time, our ability to obtain MBP-No Abx tumor samples due to recently published colorectal surgery antibiotic prophylaxis guidelines. In future studies, samples collected by colonoscopy prior to surgery may help to overcome this potential bias. Second, we analyzed a significantly higher number of colonies per tissue area from controls, and surgical specimens were also exposed to air for longer than control biopsies. These issues could have biased our results toward *bft* underrepresentation in cases. Last, our data are not as quantitative as those reported by Zitomersky et al, where terminal dilution

analysis was performed [10]. Despite these limitations, our cases were still identified to be more often *bft*-positive than our study controls.

Increasing attention is focused on understanding the contributions of colonic bacteria such as ETBF to colonic dysbiosis and human CRC [38–40]. Although *bft* is frequently detected and significantly more common in cases compared to controls herein, our results do not define exposure to biologically active BFT toxin. In addition, we did not confirm that *bft* detection was confined to *B. fragilis* sensu stricto as in prior work, nor did we define if *B. fragilis* colonization, independent of *bft* detection, differed between individuals with CRC and our control population. Many important questions remain to understand the relationship between *bft* exposure and CRC pathogenesis such as determining if mucosal and fecal *B. fragilis* and *bft* detection correlate, whether colonic inflammation correlates with *bft* detection, and/or a systemic anti-BFT antibody response. Further investigation is warranted to understand if age, sex, race, and/or diet affect *bft* detection in human populations over time.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (http://cid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No potential conflicts of interest.

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