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Original Article

Evaluation of Enterotoxigenic *Bacteroides fragilis* from Colonic Washings from Patients Undergoing Colonoscopy

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Enterotoxigenic *Bacteroides fragilis* (ETBF) is an intestinal commensal bacterium implicated as a risk factor for colon cancer. The key virulence factor is a secreted toxin called *B. fragilis* toxin (BFT). In this study we used an *in vitro* bioassay to examine the prevalence of ETBF in colonic washings from patients with colorectal polyps and normal control patients. We found that 9.3% of polyp patients and 10.9% of non-polyp patients harbored ETBF, respectively. A total of nine ETBF clinical isolates were isolated and confirmed to be positive for the BFT gene by PCR analysis and the ability to induce IL-8 secretion in the colonic epithelial cell line HT29/c1. Two of the ETBF clinical strains were characterized further *in vitro* and *in vivo*. We found that the two ETBF clinical isolates induced E-cadherin cleavage in HT29/c1 cells and promoted colonic inflammation in C57BL/6 mice. Our results indicate that the prevalence of ETBF in polyp patients were similar in non-polyp patients suggesting that ETBF carriage does not positively correlate to polyp incidence.

Key Words: Enterotoxigenic *Bacteroides fragilis*, Colon cancer, Polyps, E-cadherin, Colitis

INTRODUCTION

Colon cancer is a major cause of morbidity and mortality in the Korean population. Although much is known about the genetic pathways for colon cancer formation, the role of environmental factors such as the role of intestinal bacteria in the formation of colon cancer is less clear. *Bacteroides fragilis* are commensal bacteria residing in the human large intestine. Enterotoxigenic strains of *B. fragilis* (ETBF) are a subset of *B. fragilis* that cause inflammatory diarrheal diseases in humans and livestock (Sack et al., 1992; Sears, 2001). The key virulence factor of ETBF is a 20 kDa secreted metalloprotease called *B. fragilis* toxin (BFT) (Franco et al., 1997). This toxin induces cleavage of the epithelial junctional protein E-cadherin (Wu et al., 1998).

E-cadherins are intercellular adhesion proteins that join adjacent epithelial cells. The extracellular domain of E-cadherin of one epithelial cell interacts with E-cadherin presented on adjacent epithelial cells to form a junction while the cytoplasmic portion associates with various linker proteins. β-catenin is one such linker protein but in the absence of E-cadherin β-catenin is released from the membrane, translocates into the nucleus and activates TCF/LEF transcription factors to produce pro-cancerous proliferative proteins (c-myc and cyclin D1) and inflammatory cytokines (interleukin-8) (Wu et al., 2003; Wu et al., 2004). Activation of the β-catenin signaling pathway is a key event in polyp

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development and considered one of the major driving factors for colorectal cancers.

Numerous reports using various mouse model systems strongly suggest that persistent intestinal inflammation induced by bacteria can result in higher incidence of colon cancer (Huycke and Gaskins, 2004; Sinicrope, 2007). These and other mouse models stress the association of chronic intestinal inflammation and colorectal cancer but the molecular mechanisms remain unclear. It has been proposed that production of reactive oxygen and nitrogen species during chronic inflammation by ETBF contributes to mutagenesis (Goodwin et al., 2011). We recently developed a mouse model to study ETBF infections *in vivo* (Rhee et al., 2009). C57BL/6 mice were orally infected with ETBF strains or nontoxigenic *B. fragilis* (NTBF) strains. ETBF infected mice developed acute colitis which then transitioned into a chronic yet asymptomatic colitis lasting up to 16 months. Intestinal E-cadherin was rapidly cleaved *in vivo* with increased levels of KC (mouse homolog of IL-8) and Cyclin D1 expression by the epithelial cells. NTBF colonized mice did not develop colitis. These results demonstrate that secretion of BFT is sufficient to induce colitis.

A recent study suggested an association between ETBF colonization and colorectal cancer in humans (Toprak et al., 2006). In this study ETBF was detected in the stool of 38% of colorectal patients (21/56) compared with 12% of healthy controls (5/40). This study while limited in the number of samples examined suggests a link between ETBF and colorectal cancer. On the basis of this clinical data and in concert with the data from the ETBF mouse experiments, we hypothesize that ETBF can promote colorectal cancer in humans. To explore this hypothesis, we assessed the prevalence of ETBF in humans undergoing routine colorectal screening as a function of the presence or absence of intestinal polyps and determined whether these clinical isolates can promote colitis in a mouse model.

MATERIALS AND METHODS

Colonic washings

Colonic washings from healthy individuals undergoing routine colonoscopy were collected for ETBF identification. Colonic washings from patients were collected from February 7, 2009~October 5, 2009. All patients were approached as to participating in the ACF Database which provides permission for all clinical data to be collected anonymously, as well as for a stool sample to be obtained at the time of colonoscopy. One-half of the washings were stored as a 20% glycerol stock and the other half was used immediately for bacterial culture. The demographics of the patients were Hispanic, Caucasian and African-American. The mean age was 57.

B. fragilis culture and ETBF screening

Colonic washings were centrifuged briefly to remove debris and immediately plated on Bacteroides Bile Esculin (BBE) plates (bioMerieux, USA) to select for *Bacteroides spp*. members. After 48~72 h of growth in an anaerobic chamber, 90 well-isolated black colonies were picked with a sterile toothpick. Each individual colony was inoculated into a 96-well plate containing 100 μl of brain heart infusion broth (BHIB) supplemented with hemin, vitamin K and cysteine (Shanson and Singh, 1981; Wilkins and Chalgren, 1976). The plates were placed in an anaerobic chamber, cultured for 24 h at 37℃ and the bacterial supernatants harvested for ETBF screening.

ETBF isolates were screened by the secretion of biologically active BFT as described previously (Mundy and Sears, 1996). In brief, the bacterial supernatants were diluted 10-fold in serum-free DMEM cell culture media containing antibiotics [penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (50 μg/ml)] and overlayed onto HT29/c1 cells. Cell morphological changes (i.e., cell rounding, cell refractility) were recorded 1, 3 and 6 hours later. *B. fragilis* strains 86-5443-2-2 (BFT secreting strain) and NCTC 9343 (BFT non-secreting strain) were used as positive and negative controls, respectively (Franco et al., 2002; Myers and Shoop, 1987). Bacterial supernatants from *B. fragilis* strain 86-5443-2-2 induce cell morphology changes within 30 min whereas NCTC 9343 does not induce any changes in HT29/c1 cells for up to 48 hr. If two or more wells tested positive by this assay the patient sample was scored as ETBF positive.

PCR analysis of *BFT* gene

To confirm that the isolates exhibiting BFT activity are indeed ETBF strains harboring the *BFT* gene PCR analysis was conducted. Bacterial cultures from the 96-well plates correlating with the putative ETBF strains were transferred to a microfuge tube pelleted at 12,000 *g*. The bacterial pellet was resuspended in 100 μl of 1 M Tris-HCl (pH 7.2), boiled for 10 min and the supernatant used for PCR analysis. Primers specific for the *BFT* gene were used described previously (Franco et al., 2002). BFT negative (strain NCTC 9343) was included as negative controls. The PCR products were electrophoresed on 1.8% agarose gels and stained with ethidium bromide.

IL-8 ELISA

HT29/c1 cells were cultured with bacterial culture supernatants diluted 10-fold in serum-free DMEM for 24 h. Cell culture supernatant was harvested as saved at -20℃ until analyzed by IL-8 ELISA (R&D Systems, USA).

Western blot analysis

HT29/c1 cells were cultured with ETBF culture supernatants (1/10 dilution) and the cell lysates prepared using RIPA lysis buffer (Sigma, USA) containing a cocktail of protease inhibitors. Lysates were electrophoresed under reducing conditions on 12% SDS-PAGE gels (BioRad, USA). Proteins were transferred to a 0.22 μm nitrocellulose membrane (Protran® BA83, Whatman), probed with an N-terminal specific monoclonal antibody against E-cadherin (clone ECCD-2, Invitrogen, USA) or β-actin (Sigma, USA). After incubation with HRP-conjugated antibodies (Immuno-Research Laboratories, USA) bands were detected with SuperSignal West Pico Chemiluminescent Substrate (Pierce, USA).

Mouse infection and bacterial colonization

Specific pathogen-free (SPF) 4-week-old male C57BL/6 mice were purchased from Jackson Laboratories (USA). Experimental protocols were approved by University of Illinois Animal Care and Use Committee in accordance with the regulations of the Association for the Assessment and Accreditation of Laboratory Animal Care International. Mice infection was conducted as described previously (Rhee et al., 2009). In brief, mice were given water with clindamycin (100 mg/L) and gentamicin (300 mg/L) to decrease the normal enteric flora thus promoting ETBF colonization. Antibiotic water was initiated 7 days prior to bacterial inoculations and discontinued one day prior to bacterial inoculation. ETBF strains grown in BHIB overnight were washed with sterile PBS and adjusted to 1×10^9 colony forming units (CFU)/200 μl for mouse oral inoculations. One week post-inoculation, ETBF colonization (CFU/gram stool) was monitored microbiologically by serially dilution and plating of stool on BBE plates. *B. fragilis* were not present in baseline cultures of any mouse used in this study.

Histology and inflammation scoring

Formalin-fixed (10%), paraffin-embedded intestinal tissues were sectioned (5 μm) and stained with hematoxlyin and eosin (H&E). After one week post-infection, colonic inflammation was graded as follows: 0, normal; 1, mild increase in inflammatory cells, no mucosal epithelial changes (no proliferation or loss of crypt structure); 2, moderate increase in inflammatory cells, mild scattered mucosal epithelial proliferation ± focal loss of crypt architecture; 3, moderate increase in inflammatory cells, diffuse or nearly diffuse (>2 sites) mucosal epithelial proliferation, edema ± focal loss of crypt architecture; 4, severe increase in inflammatory cells, marked consistent proliferation; extensive loss (>2 sites) of crypt architecture; 5, complete destruction of mucosa. Images were taken using a Olympus camera and rendered using Adobe Photoshop.

RESULTS

Prevalence of ETBF in colonic washings

Colonic washings from patients undergoing routine colonoscopy were first examined for presence of polyps and then these patients stratified into two groups; 49 patients that harbored one or more polyps of >2 mm and 50 patients that did not have any polyps. The colonic washings were plated on BBE plates and single colonies of putative

Table 1. Prevalence of *Bacteroides spp* and ETBF in colonic washings

	# of Patients	*Bacteroides spp*[*].	BFT positive[**]	% ETBF[***]
With polyps	49	43 (88)	4	9.3
Without polyps	50	46 (92)	5	10.9

[*]Numbers of patients with colonic washings harboring *Bacteroides spp* as determined by growth on BBE plates. The numbers in the parentheses denote the percentage of patients that harbored *Bacteroides spp* in colonic washings.
[**]Number of patients which harbored ETBF
[***]Percentage of patients who harbored ETBF in colonic washing. Calculated as BFT positive/*Bacterodies spp*.

Fig. 1. PCR analysis of the *BFT* gene in ETBF clinical isolates. Putative ETBF clinical isolates were tested for presence of the *BFT* gene by PCR. Lane 1, *B. fragilis* 9343 (BFT negative reference strain). Lanes 2-10, ETBF clinical strains.

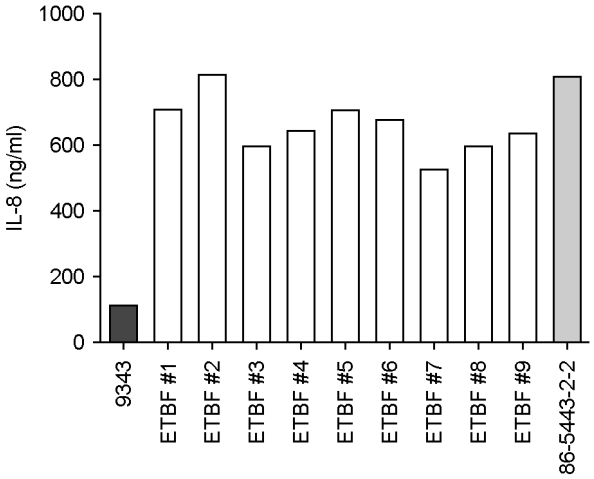

Fig. 2. ETBF clinical isolates induce IL-8 secretion. ETBF strains were cultured in BHIB for 24 hours. Filter-sterilized conditioned media (1/10 dilution) were cultured with HT29/c1 cells for 16 h and the cell culture supernatant assessed for IL-8 secretion by ELISA. Each strain was tested in triplicate wells. *B. fragilis* 9343 (BFT negative reference strain), ETBF clinical isolates #1~9 (BFT positive) and *B. fragilis* 86-5443-2-2 (BFT positive reference strain).

Bacteroides spp were picked and cultured in BHIB. Among the 49 patients with polyps, 43 patients (88%) harbored *Bacteroides spp* and among the 50 patients without polyps 46 patients (92%) harbored *Bacteroides spp*. Using the BFT bioassay we identified 4 patients with putative ETBF among the polyp patients (4/43 = 9.3%) and 5 patients with putative ETBF among the non-poly patients (5/46 = 10.9%). These results are summarized in Table 1. We confirmed that all nine putative ETBF strains identified by the bioassay harbored the *BFT* gene by PCR analysis (Fig. 1). We also confirmed that the nine ETBF clinical isolates induced IL-8 secretion in HT29/c1 cells by ELISA (Fig. 2). Taken together, these results show that all nine ETBF clinical isolates are true ETBF strains.

ETBF clinical isolates induce E-cadherin cleavage and colonic inflammation

BFT secreted by ETBF strains are known to induce cleavage of the ectodomain of E-cadherin in epithelial cell lines and colonic inflammation in mice (Rhee et al., 2009). We therefore tested whether the isolated ETBF strains also could induce E-cadherin cleavage and colonic inflammation. Two ETBF clinical isolates (#2 and #5) were selected for further study. Bacterial culture supernatants were diluted and overlayed onto HT29/c1 cells and E-cadherin cleavage examined by Western blot analysis. We found that both ETBF clinical isolates #2 and #5 induced E-cadherin cleavage whereas the non-toxigenic *B. fragilis* strain NCTC 9343 did not induce E-cadherin cleavage (Fig. 3A).

To determine if the ETBF clinical isolates could induce colonic inflammation in mice, C57BL/6 mice were infected with either the ETBF strain #2 or the ETBF strain #5. Both mice exhibited transient loss in body weight, loose stool and loss in activity consistent with previous reports (Rabizadeh et al., 2007; Rhee et al., 2009). Colonization of ETBF was was ~10^9 CFU/gram of stool (Fig. 3B). To assess colonic inflammation, the infected mice were euthanized after 7 day post-infection and the colon dissected and stained with

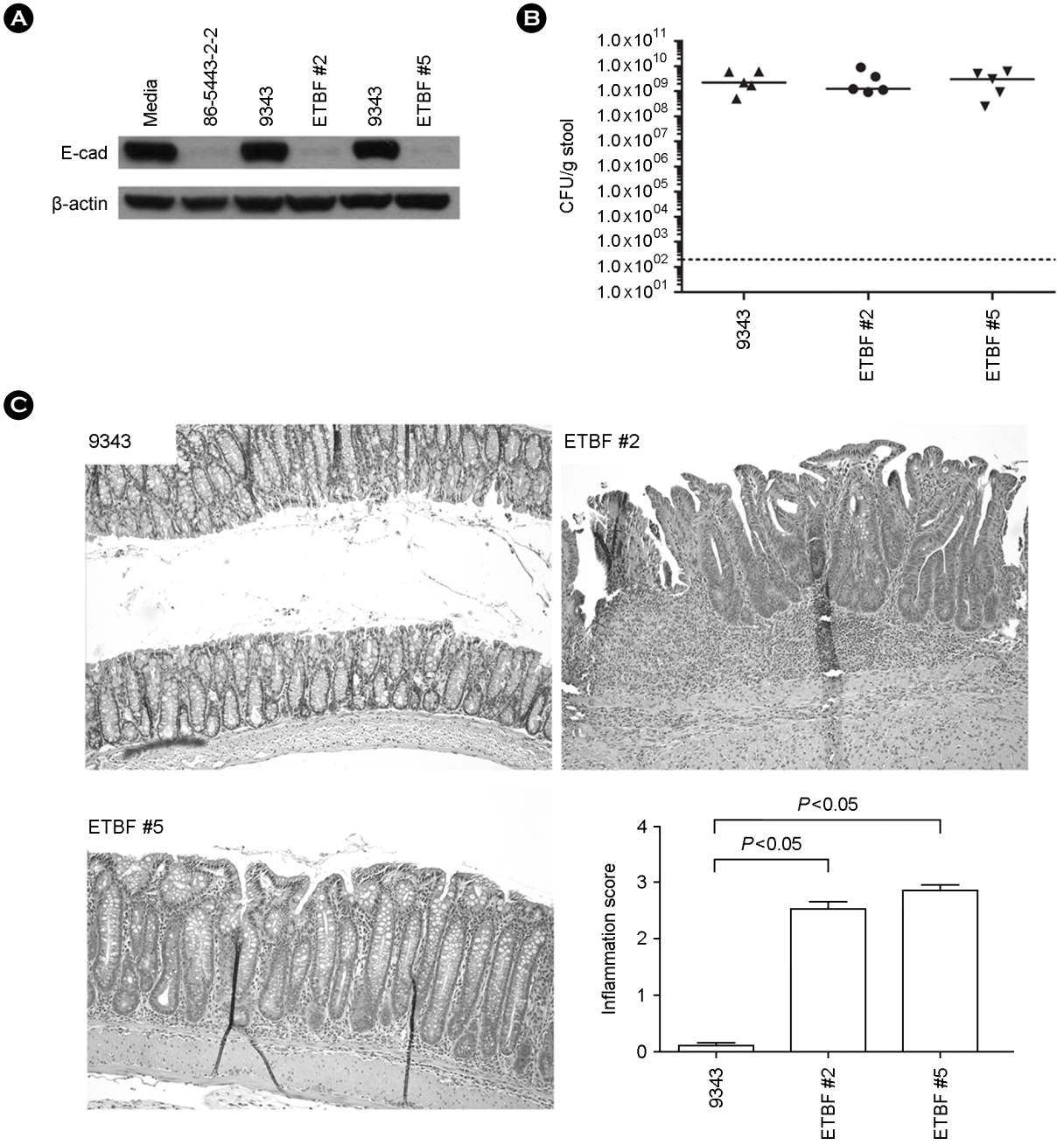

Fig. 3. ETBF clinical isolates induce E-cadherin cleavage in HT29/c1 cells and colonic inflammation in C57BL/6 mice. Panel A. Filter-sterilized conditioned media (1/10 dilution) from ETBF clinical isolate #2 and #5 were cultured with HT29/c1 cells for 3 h and E-cadherin cleavage assessed by Western blot analysis. β-actin was used as a control for equal loading. Panel B. Quantification of ETBF in stool of infected mice. C57BL/6 mice were infected with either *B. fragilis* 9343 (BFT negative), ETBF clinical isolate #2 or ETBF clinical isolate #5 for 7 days and the CFU of *B. fragilis* assessed on BBE plates. 5 mice per group. Dotted line shows the detection limit. Panel C. Hematoxylin-eosin (H&E) staining of colon tissue. H&E stained tissue sections of mice infected with *B. fragilis* 9343 (BFT negative), ETBF clinical isolate #2 or ETBF clinical isolate #5 after 7 days of infection. The histologic inflammation scores of the colon of infected mice are from 5 mice per group. *denotes lack of inflammation (inflammation score <0.2). Error bars indicate SEM. *P*, level of statistical significance using Mann-Whitney test.

H&E. We found that the ETBF clinical isolates induced epithelial hyperplasia, edema and neutrophil infiltration in the colon (Fig. 3C) whereas the non-toxigenic *B. fragilis* strain NCTC 9343 did not induce colonic inflammation.

These results demonstrate that the clinical ETBF isolates induced colonic inflammation in mice.

DISCUSSION

Several PCR based strategies have been used to detect ETBF directly from stool DNA (Akpinar et al., 2010; Avila-Campos et al., 2007; Sharma and Chaudhry, 2006). We have also conducted preliminary studies to directly detect the *BFT* gene from the colonic washings from these patients. However, we were unable to detect *BFT* gene from any of the patients examined (data not shown). This result leads us to suspect that our PCR diagnostic strategy was not detecting the low number of ETBF present in the stool. Another possibility is that the chemical composition of the colonic washings was refractive to successful DNA extraction. Another investigator currently conducting a similar study in pediatric colitis patients also has commented on the difficulty of identifying ETBF in stool using the PCR strategy (Cynthia Sears, personal communication). I therefore re-evaluated the colonic washing samples using selective bacterial growth media and a bioassay to detect BFT activity from bacterial isolates.

Data from the bioassay suggest that ~10% of patients harbor ETBF. This percentage may be an underestimate considering the lack of sensitivity compared to successful PCR based strategies using stool DNA. Furthermore, the black colonies isolated from the BBE plates are not exclusively *B. fragilis*. However, an advantage of the bioassay is that we were able not only to detect but isolate clinical ETBF isolates for further study. There are a limited number of human ETBF clinical isolates that have been extensively characterized. As a result, we have very little knowledge of the prevalence of antibiotic resistance profile, BFT activity, and genetic diversity of ETBF strains. Therefore, the isolation of nine additional ETBF strains should provide a starting point for further characterization.

Studies of ETBF carriage in colon cancer patients suggest that ETBF is more prevalent in colon cancer patients compared to normal patients (Toprak et al., 2006). Based on this report, we hypothesized that ETBF may be an etiologic agent promoting colon cancer in humans. If this hypothesis was true, we anticipated that the prevalence of ETBF would be higher in patients with polyps compared to patients without polyps. Our current data indicates that the prevalence of ETBF is similar among these two groups. However, a major drawback of our study is the limited numbers of patients. A larger, multi-institutional study is warranted to provide a better understanding of ETBF prevalence in the context of polyp presence.

REFERENCES

Akpinar M, Aktas E, Comert F, Kulah C, Sumbuloglu V. Evaluation of the prevalence of enterotoxigenic *Bacteroides fragilis* and the distribution bft gene subtypes in patients with diarrhea. Anaerobe. 2010. 16: 505-509.

Avila-Campos MJ, Liu C, Song Y, Rowlinson MC, Finegold SM. Determination of bft gene subtypes in *Bacteroides fragilis* clinical isolates. J Clin Microbiol. 2007. 45: 1336-1338.

Franco AA, Cheng RK, Goodman A, Sears CL. Modulation of bft expression by the *Bacteroides fragilis* pathogenicity island and its flanking region. Mol Microbiol. 2002. 45: 1067-1077.

Franco AA, Mundy LM, Trucksis M, Wu S, Kaper JB, Sears CL. Cloning and characterization of the *Bacteroides fragilis* metalloprotease toxin gene. Infect Immun. 1997. 65: 1007 -1013.

Huycke MM, Gaskins HR. Commensal bacteria, redox stress, and colorectal cancer: mechanisms and models. Exp Biol Med (Maywood). 2004. 229: 586-597.

Mundy LM, Sears CL. Detection of toxin production by *Bacteroides fragilis*: assay development and screening of extraintestinal clinical isolates. Clin Infect Dis. 1996. 23: 269-276.

Myers LL, Shoop DS. Association of enterotoxigenic *Bacteroides fragilis* with diarrheal disease in young pigs. Am J Vet Res. 1987. 48: 774-775.

Rabizadeh S, Rhee KJ, Wu S, Huso D, Gan CM, Golub JE, Wu X, Zhang M, Sears CL. Enterotoxigenic *Bacteroides fragilis*: A potential instigator of colitis. Inflamm Bowel Dis. 2007. 13: 1475-1483.

Rhee KJ, Wu S, Wu X, Huso DL, Karim B, Franco AA, Rabizadeh S, Golub JE, Mathews LE, Shin J, Sartor RB, Golenbock D, Hamad AR, Gan CM, Housseau F, Sears CL. Induction of persistent colitis by a human commensal, enterotoxigenic *Bacteroides fragilis*, in wild-type C57BL/6 mice. Infect Immun. 2009. 77: 1708-1718.

Sack RB, Myers LL, Almeido-Hill J, Shoop DS, Bradbury WC, Reid R, Santosham M. Enterotoxigenic *Bacteroides fragilis*: epidemiologic studies of its role as a human diarrhoeal pathogen. J Diarrhoeal Dis Res. 1992. 10: 4-9.

Sears CL. The toxins of *Bacteroides fragilis*. Toxicon. 2001. 39: 1737-1746.

Shanson DC, Singh J. Effect of adding cysteine to brain-heart infusion broth on the isolation of *Bacteroides fragilis* from experimental blood cultures. J Clin Pathol. 1981. 34: 221-223.

Sharma N, Chaudhry R. Rapid detection of enterotoxigenic *Bacteroides fragilis* in diarrhoeal faecal samples. Indian J Med Res. 2006. 124: 575-582.

Sinicrope FA. Sporadic colorectal cancer: an infectious disease? Gastroenterology. 2007. 132: 797-801.

Toprak NU, Yagci A, Gulluoglu BM, Akin ML, Demirkalem P, Celenk T, Soyletir G. A possible role of *Bacteroides fragilis* enterotoxin in the aetiology of colorectal cancer. Clin Microbiol Infect. 2006. 12: 782-786.

Wilkins TD, Chalgren S. Medium for use in antibiotic susceptibility testing of anaerobic bacteria. Antimicrob Agents Chemother. 1976. 10: 926-928.

Wu S, Lim KC, Huang J, Saidi RF, Sears CL. *Bacteroides fragilis* enterotoxin cleaves the zonula adherens protein, E-cadherin. Proc Natl Acad Sci U S A. 1998. 95: 14979-14984.

Wu S, Morin PJ, Maouyo D, Sears CL. *Bacteroides fragilis* enterotoxin induces c-Myc expression and cellular proliferation. Gastroenterology. 2003. 124: 392-400.

Wu S, Powell J, Mathioudakis N, Kane S, Fernandez E, Sears CL. *Bacteroides fragilis* enterotoxin induces intestinal epithelial cell secretion of interleukin-8 through mitogen-activated protein kinases and a tyrosine kinase-regulated nuclear factor-κB pathway. Infect Immun. 2004. 72: 5832-5839.