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Full Paper

Clostridioides difficile antibody response of colorectal cancer patients versus clinically healthy individuals

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Dysbiosis, defined as an imbalance in the gut microbiota caused by too few beneficial bacteria and an overgrowth of bad bacteria, yeast, and/or parasites, is now being associated with several diseases, including the development of colorectal carcinoma (CRC). In this study, the potential association of *Clostridioides difficile* (formerly *Clostridium difficile*) with CRC was investigated. Plasma samples obtained from preoperative histologically confirmed CRC patients (*n*=39) and their age- and sex-matched clinically healthy controls (*n*=39) were analyzed for antibodies to toxin B of *C. difficile* (anti-tcdB) by enzyme-linked immunosorbent assay (ELISA). A significantly greater number (p=0.012) of CRC cases (*n*=26/39, 66.7%) had anti-tcdB IgG levels above the cutoff value compared with controls (*n*=12/39, 30.8%). Eight cases (8/39, 20.5%) and none of the controls registered anti-tcdB IgA levels above the cutoff value (p=0.0039). Anti-tcdB IgG and IgA levels were not shown to be significantly associated with tumor grade or tumor stage. Anti-tcdB IgG showed 66.7% sensitivity and 69.2% specificity. For anti-tcdB IgA, sensitivity and specificity were 20.5% and 100%, respectively. The positive predictive values for anti-tcdB IgA and IgG were 100% and 68.4%, respectively. The anti-tcdB IgA and IgG negative predictive values were 55.7% and 67.5%, respectively. The results suggest the potential association of *C. difficile* with CRC and anti-tcdB levels, particularly the IgA level. Hence, anti-tcdB antibodies can be candidate serologic markers for CRC.

Key words: colorectal cancer, *Clostridioides difficile*, ELISA, dysbiosis, gut microbiome, tcdB, diagnostic performance

INTRODUCTION

Colorectal cancer (CRC) is one of the most prevalent cancers in the world [1]. Diet, particularly the consumption of poultry and animal products, has been linked to increased cases of CRC [2]. Imbalance in chromosome number, genomic amplifications in the subchromosomal region, and high frequency of heterozygous loss also lead to mutation and malignant transition of cells in the colon [3, 4].

In addition, the potential role of gut microbes in CRC development has been a hot topic lately. Several hypotheses have emerged on how these bacteria promote carcinogenesis. Dysbiosis and alterations in the normal microbial community remodel the whole microbiome, initiating inflammation and cell differentiation which could eventually lead to cancer [5]. The "driver-passenger" model proposes that some bacteria classified as "bacterial drivers" initiate the development of colonic tumors through gene damage, stimulating the colonization of passenger bacteria in the tumoral microenvironment [5, 6]. Some "keystone pathogens" that emerge during dysbiosis and are likely to be part of carcinogenesis include *Bacteroides*, *Enterococcus*, *Fusobacterium, Streptococcus, Escherichia coli,* and *Clostridium* [5, 7].

*Clostridioides difficile (*formerly *Clostridium difficile)* is a gram-positive, anaerobic, motile endospore-forming bacterium that is part of the normal gut microbiota and present in 2–5% of the adult population [8]. Few studies have been conducted on the potential role of *C. difficile* in the malignant transformation of cells in the colon [9–12]. Supposedly, the toxin B of *C. difficile* (tcdB) deregulates *Rho*-GTPases, leading to increased expression

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of proto-oncogenes [13]. Hence, this study was conducted to determine any association of *C. difficile* with CRC development among preoperative Filipino patients by analyzing their antibody levels against tcdB.

MATERIALS AND METHODS

This study was approved by the Institutional Review Boards of the University of Santo Tomas Hospital (USTH) in Manila (IRB-2016-11-191-IS-A1/CR2) and Mariano Marcos Memorial Hospital and Medical Center (MMMHMC) in Ilocos Norte (RERC-17-001). All participants gave their written informed consent.

Preoperative patients with histologically confirmed CRC (n=39) seen at USTH and MMMHMC between April 2018 and March 2019 were enrolled as cases. They were age (± 2 years) and sex-matched with physician-assessed clinically healthy controls (n=39) living in the same locality. All clinical data were retrieved from medical records. Blood was collected in EDTA tubes from all participants. Plasma was immediately separated by centrifugation at 2,500 RPM for 15 min and stored at −20°C until analysis.

Plasma samples were analyzed for anti-tcdB IgG and IgA using a commercial enzyme-linked immunosorbent assay (ELISA; tgcBiomics, Bingen am Rhein, Germany) according to the manufacturer's protocol. Negative and positive controls provided by the manufacturer were run in parallel with the samples for each plate. Absorbance readings equal to or above the cutoff value ($OD_{450-620}$=0.200) as set by the manufacturer were considered positive. Each sample was analyzed in duplicate, and ELISA was run twice to assess reproducibility of results.

Anti-tcdB IgG and IgA levels of CRC cases and matched clinically healthy controls were compared using paired t-test, and $p<0.05$ was considered significant. Logistic regression analyses followed by one-way ANOVA and two-sample t-test with equal variances were performed to determine any association of anti-tcdB IgG or IgA levels with tumor grade and tumor stage, respectively. Diagnostic performance (sensitivity, specificity, positive and negative predictive values) of the anti-tcdB ELISA was also computed. All statistical analyses were conducted using the Stata 14 software (StataCorp, College Station, TX, USA).

RESULTS

Clinical and serologic profiles of CRC cases and matched clinically healthy controls

There were more males (22/39, 56.4%) than females, and the mean age at initial diagnosis was 58 years old. Most of the cases had well (28.2%) or moderately (35.9%) differentiated tumors and were in an advanced stage (stage III or IV, 48.7%) at presentation (Table 1).

Significantly higher numbers of CRC cases had anti-tcdB IgG (p=0.012) and IgA (p=0.004) levels above the cutoff values compared with controls. Among the study participants, 26 (66.7%) cases and 12 (30.8%) controls tested positive for the anti-tcdB IgG. For IgA, only 8 (20.8%) of the cases and none of the controls were positive. Mean absorbances of the cases (IgG=0.520; IgA=0.171) were significantly higher (p=0.0041 for IgG; p=0.0051 for IgA) than those of the controls (IgG=0.260; IgA=0.073; Fig. 1).

Association of anti-tcdB IgG and IgA levels with tumor grade and stage

Mean anti-tcdB IgG and IgA levels of CRC cases with well-

Table 1. Clinical characteristics of the cases

Characteristics	n=39	%
Sex		
Male	22	56.4
Female	17	43.6
Age at initial diagnosis		
<50 years	11	28.2
≥50 years	28	71.8
Mean age at initial diagnosis (years ± SD)	58 ± 12	
Median age at initial diagnosis (years ± SD)	61 ± 12	
Tumor grade		
G1 (well-differentiated)	11	28.2
G2 (moderately-differentiated)	14	35.9
G3 (poorly-differentiated)	3	7.7
No information available	11	28.2
Tumor stage		
T1/2 (early)	8	20.5
III/IV (advanced)	19	48.7
No information available	12	30.8
Tumor location		
Colon	17	43.6
Rectum	17	43.6
Rectosigmoid	3	7.7
Cecum	1	2.6
Sigmoid	1	2.6

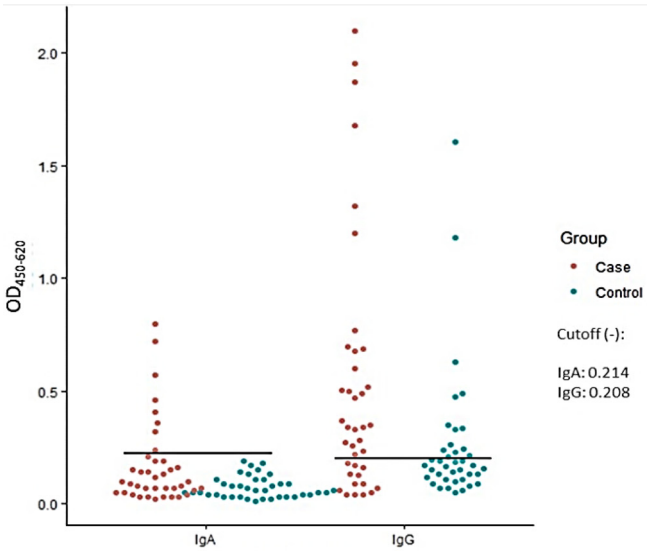

Fig. 1. Anti-tcdB IgG and IgA levels of CRC patients versus healthy controls. Significantly higher numbers of CRC cases had anti-tcdB IgG (p=0.012) and IgA (p=0.004) titers above the cutoff values compared with controls. The mean IgG absorbance of the cases (0.520) was significantly higher (p=0.0041) than that of the controls (0.260). The mean IgA absorbance of the cases (0.171) was also significantly higher (p=0.0051) than that of the controls (0.073).

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Table 2. Association of tumor grade and tumor stage with anti-tcdB levels

Tumor grade	n=28	Mean IgG	SD	p-value*	Mean IgA titer	SD	p-value*
Well-differentiated	11	0.54	0.46	0.5337	0.21	0.23	0.7506
Moderately differentiated	14	0.63	0.64		0.19	0.22	
Poorly differentiated	3	0.23	0.25		0.10	0.04	
Tumor stage	n=27	Mean IgG	SD	p-value#	Mean IgA titer	SD	p-value#
Early stages (I/II)	8	0.62	0.63	0.4845	0.22	0.27	0.2522
Advanced stages (III/IV)	19	0.46	0.48		0.13	0.11	

SD: standard deviation. *One-way ANOVA. #Two-sample t-test with equal variances.
It should be noted that information was not available for the tumor stages and grades of 11 and 12 cases, respectively.

differentiated, moderately differentiated, and poorly differentiated tumors were not significantly different (IgG, p=0.5337; IgA, p=0.7506) from each other. Likewise, those diagnosed with early (I/II) stages of CRC had mean anti-tcdB IgG and IgA levels that were not significantly (IgG, p=0.4845; IgA, p=0.2522) different from those who presented at an advanced (III/IV) stage of the disease (Table 2).

Diagnostic value of anti-tcdB IgG and IgA levels

The performance of the anti-tcdB ELISA in discriminating CRC was computed using the histologically confirmed CRC cases who tested positive for anti-tcdB IgG or IgA as true positives and the physician-assessed clinically healthy and malignancy-free volunteer controls who tested negative for anti-tcdB IgG or IgA as true negatives. Table 3 shows that anti-tcdB IgG is fairly sensitive (66.7%) and moderately specific (69.2%) for detection of CRC. Meanwhile, anti-tcdB IgA was very specific (100%) but showed very low sensitivity (20.5%). Since the positive predictive value for anti-tcdB IgA was 100%, a positive test could be equated with having CRC. Since the negative predictive value was 55.7%, a negative or normal test for anti-tcdB IgA would require other tests to confirm the results.

Table 3. Diagnostic performance of anti-tcdB levels in detecting colorectal carcinoma

Parameters*	IgG (%)	IgA (%)
Sensitivity	66.7	20.5
Specificity	69.2	100.0
Positive predictive value	68.4	100.0
Negative predictive value	67.5	55.7

*At 95% confidence interval (CI).

DISCUSSION

The results of this study show that a significantly higher number of preoperative histologically confirmed CRC cases were positive for the anti-tcdB IgG and IgA antibodies compared with their age- and sex-matched clinically healthy controls. Based on the above observations, it can be inferred that there is a potential association of *C. difficile* with CRC among selected Filipino patients.

A high prevalence of *C. difficile* among preoperative CRC patients was previously reported in China [10]. It was also observed that a significantly higher quantity of *Fusobacterium nucleatum* and *C. difficile* were present in fecal samples of CRC patients compared with healthy controls in Brazil, suggesting that these bacteria may play a significant role in colon carcinogenesis [9]. Whole-genome sequencing of the gut microbiota further showed that *Bacteroides, Fusobacterium, Streptococcus,* and *Clostridium* were the most abundant genera in tumor versus normal samples [12]. Another study unexpectedly discovered that *Clostridium*, *Fusobacterium,* and *Lactobacillus* species were more abundant in the gut than *Helicobacter pylori.* The study also found that these bacteria demonstrated certain cancer-specific bacterial signatures [11].

Members of the human intestinal microbiota have been implicated in the development of CRC. Enterotoxigenic *Bacteroides fragilis* (ETBF) induces colonic tumors by triggering a Th17 inflammatory response [14] and activating signal transducer and activator of transcription 3 (STAT3) [15]. *F. nucleatum* is thought to promote inflammation and tumorigenesis by modulating the tumor immune microenvironment via expansion of myeloid-derived immune cells [16]. A study found intraepithelial *E. coli* in the tumors of CRC cases, specifically in the colonic mucosa [17]. The prevalence of *E. coli* in the colon could have induced chronic inflammatory responses [18], contributing to CRC development, as has been observed [19]. *Streptococcus gallolyticus* subsp. *gallolyticus* has also been demonstrated to promote malignant transformation of colon cells depending on cell context, bacterial growth phase, and direct contact between bacteria and colon cancer cells [20–22].

The potent toxin B enterotoxin of *C. difficile* (tcdB) has been proven to induce the inflammatory response that occurs in pseudomembranous colitis [23]. First, it binds to the receptors of the target-specific host cells when released into the environment. The toxin-receptor complex is endocytosed into the host cells, and the toxin is translocated into the cytosol, passing through the acidic endosomal membrane. As the active toxin moiety (glucosyltransferase) is released into the environment, it transfers glucose into the *Rho* proteases, preventing the normal binding of GTP to the GDP-bound form of *Rho* protein, thus making it inactive. Any deregulation of the activities of these proteins promotes pathogenic effects such as the disruption of epithelial integrity and opening of tight junctions [24]. Immune mechanisms such as vascular permeability, tumor necrosis α activation, and pro-inflammatory interleukin production are then stimulated, leading to chronic inflammation-induced colitis and inflammatory bowel disease, which are major risk factors for growth and proliferation of tumor cells in the colorectal tract [25–27].

C. difficile has been proven to proliferate during antibiotic-induced dysbiosis in the gut microenvironment [28–31]. Antibiotic treatments deplete the commensal bacteria in the gut,

which are known to metabolize primary bile acids into secondary bile acids that, in turn, inhibit the proliferation of *C. difficile*. Hence, depletion of commensal bacteria leads to accumulation of primary bile acids, which serve as energy sources of *C. difficile* for growth and proliferation [32].

Records show that the Philippines has a history of prevalent antibiotics misuse [33] and self-medication [34]. Antibiotics are widely available in convenience stores, and Filipinos have been observed to often share them with family members, even those without prescriptions [33]. Moreover, agriculture, aquaculture, and horticulture businesses in the Philippines commonly incorporate antibiotics in their products [35]. Hence, a longitudinal study of long-term antibiotic use in patients answer questions related to antibiotic-induced *C. difficile* proliferation and CRC development.

While previous studies [9, 10] analyzed fecal samples by molecular techniques, the current study compared the anti-tcdB IgG and IgA levels of preoperative CRC patients with matched physician-assessed clinically healthy controls. The results show that the anti-tcdB IgA titer was more associated with CRC than the anti-tcdB IgG titer and hence was a more valuable serologic marker. The elevated anti-tcdB IgA levels may be attributed to the invasion of the mucosal barrier of the colon or rectum by *C. difficile* [36]. Meanwhile, the presence of this bacterium in 2–5% of the adult population [8] may explain why a number of clinically healthy cases also tested positive for anti-tcdB IgG.

Similarly, higher levels of anti-*F. nucleatum* (anti-Fn) IgA have been recorded in preoperative CRC patients compared with healthy controls [37]. In the current study, anti-tcdB IgG and IgA levels were not seen to be associated with the tumor stage or tumor grade. In comparison, anti-Fn IgG, but not IgA, levels were associated with the tumor grade [37]. But similar to the current study, tumor stage was not also associated with anti-Fn IgG or IgA levels [37]. Serologic markers have been useful in evaluating other infection-associated cancers, such as those caused by the human papillomavirus [38, 39], Epstein Barr virus [40], and *H. pylori* [41].

While the results show that a significantly greater number of preoperative CRC patients were positive for the anti-tcdB antibodies, it is not certain whether this organism directly or indirectly induced the malignant transformation of colon cells. It could be that the increase in anti-tcdB IgG and IgA levels was due to chronic infection with *C. difficile* or that the presence of malignancy weakened the beneficial members of the gut microbiota, thereby increasing the risk of *C. difficile* colonizing the large intestine. Whichever is the underlying reason, anti-tcdB antibodies have proven to be valuable in evaluating CRC.

This study contributes to the limited data on the high occurrence of *C. difficile* in CRC cases. However, it was only able to enroll a small number of preoperative CRC cases, since the majority of the patients from the study sites had already undergone surgery and other forms of treatment when the study was initiated. Future studies should include information on long-term antibiotics use and history of inflammatory bowel disease. Molecular analysis of tissues and fecal samples must also be done to confirm the results of the current study. Finally, assays for carcinoembryonic antigen (CEA) and CA 19-9 can be run in parallel with anti-tcdB IgG and IgA to determine if the sensitivity of the latter can be significantly improved without compromising the specificity, as has been observed with anti-Fn antibodies [37].

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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