Clostridioides difficile Toxin B (tcdB) Peptide Mimotope Antibody-capture Enzyme Immunoassay in the Detection of Colorectal Cancer

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ABSTRACT

BACKGROUND: The role of certain gut microbes in the development of colorectal cancer has recently been understood. In our previous work, we have reported the reactivity of serum collected from CRC patients to peptide mimotopes of bacterial antigens associated with the development of CRC, hence, their potential in its detection. Here, we evaluate the diagnostic parameters of an anti-*Clostridioides difficile* toxin B (tcdB) peptide mimotope IgG and IgA antibody-capture enzyme-linked immunosorbent assay (ELISA) in the detection of CRC.

METHODS: Prediction of the immunogenic epitopes of tcdB was made using the *in silico* B-cell epitope mapping tools available at the Immune Epitope Database and Analysis Resources (IEDB). Synthetic peptide analogs of the predicted epitopes which were used in the immunoassays were synthesized and purchased from Genscript (New Jersey, USA). Presence of anti-peptide IgG and IgA from serum collected from patients diagnosed with colorectal cancer were detected by a peptide antibody-capture indirect ELISA protocol developed in this study. Diagnostic parameters were computed.

RESULTS: The amino acid position 1465 to 1474 of the reported sequence of tcdB (PDB Accession No. AGG91641.1) was predicted to be immunogenic. The peptide antibody-capture ELISA developed in this study gave diagnostic specificities of 94.9% and 97.4%, positive predictive values (PPV) of 86.6% and 93.3%, and likelihood ratios of 6.5 and 14.0 for IgG and IgA, respectively. Our results show the potential of the immunoassay designed in ruling in possibility of CRC during diagnosis.

Keywords: peptide ELISA, *C. difficile* toxin B (tcdB), colorectal cancer, B-cell epitope

BACKGROUND

Colorectal cancer (CRC) is the third highest in incidence and the second highest in mortality rate in the world. In its 2018 report, the World Health Organization (WHO) reported 1.8 million cases of and 862 thousand deaths due to CRC [1]. Alarmingly, this rate has been observed to increase over the last 25 years with the highest rate of increase observed among the young adults [2].

CRC has been associated with many factors which include genetic, environmental, and modifiable lifestyle behaviors [3, 4]. Hereditary colorectal cancer which is attributed to 5-10% of CRC, for example, has been associated with mutations in the tumor suppressor gene adenomatous polyposis coli (*APC*) gene and several mutation repair genes such as the MutL homolog1 (*MLH1*) and MutS homologs 2 and 6 (*MSH2* and *MSH6*) [4]. It has also been associated with occupational related hazards due to exposure to cancer-causing agents [5]. Several lifestyle behaviors such as smoking, alcohol intake, diet and physical activities have also been implicated in the incidence of CRC [6, 7]. Recently, several gut microbes and the toxins they produced have been implicated in colorectal cancer tumorigenesis [8, 9]. One of them is *Clostridioides* (formerly known as Clostridium) *difficile* [10].

C. difficile is a gram-positive, anaerobic opportunistic microbe that is associated with the development of CRC [11]. This spore-forming microbe colonizes the colon and secretes intestinal cell epithelium-damaging toxins tcdA and tcdB [9, 12]. Of the two, only tcdB is reported to have the ability to disrupt epithelial cell integrity due to the systemic damage it causes to the crypt base of the colonic epithelium which causes the consistent recruitment of inflammatory response affecting cell-signaling and homeostasis, a brewing condition for carcinogenesis [13, 14]. This well explained mechanism of bacterial toxin-initiated tumorigenesis provides the framework and

basis for the tcdB, and antibodies produced by infected individuals, as a biomarker for colorectal cancer [10].

In this study, we examined the clinical diagnostic parameters of an anti-tcdB IgG and IgA capture ELISA we designed to detect CRC.

METHODOLOGY

Sample Population

Patients born from Filipino parents, 18 years old and above, and with histologically confirmed colorectal cancer (CRC) seen at USTH and MMMH-MC between April 2018 and March 2019 and have not undergone any form of treatment for their cancer were enrolled as cases in the study. The cases were age (±2 years) and sex-matched with volunteer controls living in the same locality. The controls were assessed by a licensed physician as clinically healthy and not suspected of any malignancy. Clinical data of the cases such as the age at initial diagnosis, tumor grade, tumor stage, and type of CRC were retrieved from histopathology reports and medical records. Blood was collected in EDTA tubes from all study participants. Plasma was immediately separated by centrifugation at 2500 RPM for 15 minutes and stored at -20°C until serologic analysis.

In-silico Epitope Prediction of tcdB

The protein sequence of *Clostridioides difficile* toxin B (tcdB) was acquired from the National Center for Biotechnology Information Protein Database (NCBI-PDB). Immune epitopes of the reported sequence of *C. difficile* tcdB were predicted using the B-cell epitope prediction analysis resource of the Immune Epitope Database (IEDB) and Analysis Resources. The tcdB

epitopes were chosen based on the antigenicity, hydrophilicity, and surface accessibility scores using the Kolaskar and Tongaonkar Antigenicity Prediction, Parker Hydrophilicity Prediction and Emini Surface Accessibility Prediction tools, respectively. The sequence with the highest antigenic score and hydrophilicity and surface accessibility scores higher than the generated threshold for hydrophilicity and surface accessibility scores above the threshold values was selected for synthesis and use in this study. Synthetic peptide analog of this predicted epitope was synthesized by and purchased from GenScript Corporation (New Jersey, USA).

Chessboard Titration Method

Initially, all samples were tested for anti-tcdB IgG and IgA titers using a commercial enzyme-linked immunosorbent assay kit (ELISA; tgcBiomics, Bingen am Rhein, Germany). Samples that tested positive for IgG (*n*=38) and IgA (*n*=8) were pooled and used in the optimization process through chessboard titration [15].

The lyophilized tcdB peptide was first reconstituted with 1x PBS (pH 7.4) to a concentration of 4.3 mg/mL following the manufacturer's protocol (GenScript, USA). The reconstituted tcdB peptide was diluted with bicarbonate/carbonate coating buffer (pH 9.6) to concentrations of 50, 10, 5, 1, 0.5, and 0.1 µg/mL. Pooled plasma samples were diluted to 1:100, 1:200, and 1:400 and the HRP-conjugated anti-IgG and anti-IgA with concentration of 1.2 mg/mL (Abcam, USA) were diluted to 1:500 and 1:1000. ELISA of the pooled samples were performed with the tcdB peptide coating concentration (µg/mL), plasma sample dilution, and HRP-conjugated secondary antibody dilution tested simultaneously [16]. The optimum working conditions were decided based on the combined lowest concentrations of the different components that gave strongest signal versus low background.

Analysis of CRC Cases and Clinically Healthy Controls

Fifty (50) µl of the optimized tcdB concentration was added to the assigned wells of a 96-well microtiter plate and incubated for 16-18 hr at 4ºC. After incubation, the wells were washed thrice with phosphate buffered saline-0.05% Tweenº20 (PBS-T, pH 7.4). All 96 wells were blocked with 100 µl of 1% BSA in PBS-T (pH 7.4), incubated for 60 min at room temperature and washed thrice.

The plasma samples of the CRC cases and their matched controls were diluted with 1% BSA in PBS-T (pH 7.4). Fifty (50) µl of the optimized primary antibody dilution was transferred into the assigned wells, incubated for 60 min at room temperature, and then washed thrice. Fifty microliters of diluted HRP-conjugated anti-human IgG (Invitrogen, USA) was added to the specified wells, incubated for 60 min at room temperature, and washed thrice.

All 96 wells were added with 50 µl TMB substrate solution (Sigma-Aldrich), incubated in the dark at room temperature until color development (approximately 5 min). Fifty (50) µl 2.5M H_2SO_4 was added to all wells to stop the reaction. Absorbance was read at 450 nm and the mean OD of two blank wells was subtracted from the OD of each sample. The above-mentioned steps were repeated for analysis of IgA antibodies, with HRP-conjugated anti-human IgA (Invitrogen, USA) as secondary or detection antibodies. The cut-off values were computed based on the formula described by Frey et al. (1998) [17].

Data Analysis

Absorbance values equal to or above the respective cut-off value (COV) were considered positive or reactive, while all values below COV were considered negative or non-reactive. Paired

t-test was used to compare the anti-tcdB IgG and IgA titers of CRC cases and controls while Pearson's correlation was performed to determine any association of antibody levels with tumor grade and stage. *P*-values *<0.05* were considered significant. Diagnostic performance of the tcdB peptide ELISA was computed using the histologic diagnosis as gold standard. All statistical analyses were conducted using Microsoft Excel and MATLAB R2020b.

RESULTS

Clinical Characteristics of Cases

Thirty-nine (39) histologically confirmed CRC cases who have not undergone any form of treatment for their condition and 39 age- and sex-matched clinically healthy controls seen at USTH and MMMH-MC between April 2018 and March 2019 were enrolled in the study. Majority of the cases were males (n=22, *56.4%*). The mean and median ages at initial diagnosis were 58 and 61 years old, respectively. Of the 39 cases, 11, 14, and 3 had well, moderately, and poorly differentiated tumors, respectively. Nineteen (19) cases were in the advanced stage of the disease at presentation. Information on their tumor grade and stage were not available for 11 and 12 of the cases, respectively.

In-silico Epitope Mapping

The amino acid sequence tcdB used in this study is the reported sequence with PDB Accession No. AGG91641.1. [18]. The amino acid sequence VDSEGKENGF located at position 1465 to 1474 yields an antigenicity score of 0.95 against a threshold of 0.92, hydrophilicity score of 4.33 against a threshold of 3.63 and surface accessibility score of 1.26 against a threshold of 1.00. Since this peptide have scores higher than the computed cut-off, a peptide analog of this

predicted epitope was requested for synthesis to Genscript Corporation. Analysis of the peptide synthesized, the peptide used in the immunoassays done in the succeeding experiments, is 90.7% purity by HPLC and its solubility is greater than 5mg/mL in ultrapure water, 1x DPBS (pH 7.1 ± 0.1), and dimethylsulfoxide (DMSO).

Analysis of samples

The optimum working conditions for the tcdB peptide ELISA were 0.1 µg/mL for the coating peptide, 1:100 plasma dilution, and 1:1000 detection antibody dilution. Cut-off values for anti-tcdB IgA and IgG were 0.74 and 0.60, respectively. A significantly higher number of CRC cases tested positive for anti-tcdB IgG (P<0.001) and IgA (P<0.001) compared to controls. Among the study participants, 13 CRC cases and 2 controls were tcdB IgG-reactive. Meanwhile, 14 CRC cases and only 1 control tested positive to tcdB IgA. The mean tcdB IgG (0.5174) and tcdB IgA (0.6018) titers of the cases were significantly higher (P<0.0001) than the controls (IgG=0.3423; IgA=0.3841) (Figure 1). Similar to the commercial kit used in our previous study [15], this in-house developed assay was not able to identify any correlation between antibody levels and tumor stage (IgG r=-0.111; P=0.566 and IgA r=0.175; P=0.365) or tumor grade (IgG r=-0.243; P=0.213 and IgA r=0.185; P=0.346).

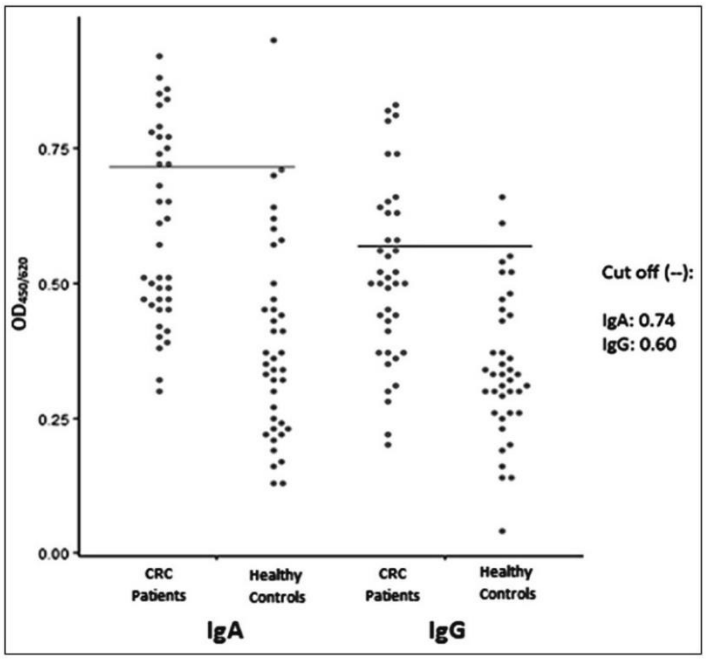

Figure 01. Anti-tcdB IgG and IgA titers using in-house developed ELISA. Significantly higher number of CRC cases had anti-tcdB IgG (*p<0.001*) and anti-tcdB IgA (*p<0.001*) titers above cut-off values compared to controls.

Diagnostic performance of the peptide ELISA

Diagnostic performance of the peptide ELISA was computed using the histopathologic diagnosis as gold standard. The assay appears highly specific (IgG=94.9%; IgA=97.4%) but not sensitive (IgG=33.3%; IgA=35.9%), hence affecting the accuracy of the test (IgG=64.1%; IgA=66.7%). Between anti-tcdB IgA and IgG, the former provides the higher likelihood (PPV=93.3%) that a patient has CRC. However, neither tcdB IgA (NPV=60.3%) nor IgG (NPV=58.7%) is a good measure that a person has no CRC. With an LR+ value of 14, tcdB IgA appears to be a good indicator for ruling-in diagnosis [19]. Moreover, the computed area under the curve (AUC) of the tcdB IgA (0.82) suggests that it is a better diagnostic test compared to tcdB IgG (0.79). Table 1 summarizes the diagnostic performance of the in-house peptide ELISA.

Table 1. Diagnostic performance of the in-house developed peptide ELISA

Performance Parameter	IgG	IgA
Specificity	94.9%	97.4%
Sensitivity	33.3%	35.9%
Accuracy	64.1%	66.7%
Positive Predictive Value	86.6%	93.3%
Negative Predictive Value	58.7	60.3
AUC	0.79	0.82

DISCUSSION

While current serologic tests detect tumor-related antigens as the biomarker for cancer, the use of antibodies against these antigens for cancer detection have already been studied as early as the 1990s [20, 21]. This method has been reported to give higher specificity because of its ability to distinguish between presence of tumor markers due to cancer and inflammation [21]. In this study, we combined *in silico* epitope prediction approaches and *in vitro* assays to show that, given the well-understood mechanism of bacterial toxin-associated CRC tumorigenesis, antibodies against specific epitopes of tcdB can also be used in the detection of colorectal cancer.

The epitopes were identified based on the predicted physico-chemical properties of the given linear peptide sequences. This includes antigenicity, surface accessibility and hydrophilicity, typical characteristics of linear immunogenic epitopes of antigens [22]. The epitope mapping tools used in this study have previously been used to identify immunogenic sequences of known antigens from infectious agents and cancer biomarkers [23, 24]. Its applications ranged from vaccine research to diagnostic tool development, from which the outcomes for the latter is improvement in the sensitivity, specificity, or both in detection of diseases [25-26].

The availability of bioinformatics techniques has shown to be a very powerful tool to identify the immunogenic epitopes in this study. Bioinformatics tools specifically designed to predict immunogenic portions of antigens is an efficient method of epitope mapping, an important step in vaccine and immunodiagnostics research. The ability to identify possible immunogenic sequences using *in silico* approaches provided a bypass highway in vaccine and diagnostic research [27]. It eliminated the costly and inefficient method of hydrolyzing antigens at the site of basic or aromatic amino acid residues which frequently play a role in the immunogenicity of certain sequences [28, 29]. Together with synthetic peptide technology, it made possible high throughput testing without the tedious process of isolation and purification of randomly selected peptide sequences from the actual antigen sources [30].

We used synthetic peptides comprising the amino acid sequence of the predicted immunogenic epitope to capture anti-tcdB IgG and IgA in this study. The synthetic peptide was used as a mimotope of the actual tcdB epitope in capturing anti-tcdB epitope-specific antibodies which, in our previous study, have been shown as a potential biomarker of CRC [15]. This ability of synthetic peptides to capture antibodies is attributed to their ability to mimic the structure of the immunogenic epitopes in the native antigen molecule and serve its function in diagnosis of diseases [31, 32]. This approach represents a promising platform in using peptides for molecular diagnostics and therapeutics [33].

The high percentage of true positives which contributed to the high specificity and high PPV can also be attributed to the efficiency of peptide epitopes in binding epitope-specific antibodies during detection. This has been reported in previous studies both in infectious and cancer diagnostics [34, 35]. However, we may attribute the low sensitivity to the multifaceted

nature of colorectal cancer [36]. Since the assay is only intended to detect anti-tcdB IgA and IgG, the false negatives can be explained by non-tcdB related CRC.

tcdB-induced CRC is only one among the many probable causes of cancer [3, 9]. Genetic and environmental factors or other CRC-associated pathogens and not *C. difficile* infection may have induced the development of cancer which caused the false negative results in an anti-tcdB IgG and IgA detection test for CRC contributing to low sensitivity [4, 5].

Our results also show that anti-tcdB IgA titer was more associated with CRC than anti-tcdB IgG, hence a better serologic marker. A possible explanation to this is that IgA is a product of local inflammation and it constitutes the major class of antibodies at any mucosal surface, and in addition, the most heavily produced immunoglobulin (66 mg/kg/day) and second most prevalent circulating antibody besides IgG [37, 38].

CONCLUSION

We were able to identify the diagnostic parameters of the tcdB peptide mimotope IgA and IgG antibody capture ELISA developed in this study. The high specificity and PPV of the IgA and IgG shows the potential of the method developed in the ruling of presence of CRC during diagnosis.

LIST OF ABBREVIATIONS

APC - adenomatous polyposis coli

COV - cut-off value

CRC - colorectal cancer

ELISA - Enzyme-linked Immunosorbent Assay

IEDB - Immune Epitope Database and Analysis Resources

IgA - Immunoglobulin A

IgG - Immunoglobulin G

MLH1 - MutL homolog 1

MSH2 - MutS homolog 2

MSH6 - MutS homolog 6

NCBI-PDB- National Center for Biotechnology Information Protein Database

NPV - negative predictive value

PPV - positive predictive value

tcdB - *Clostridioides difficile* Toxin B

DECLARATIONS

Ethical Statement

All study protocols, research design, and other pertinent documents were reviewed and approved by the Institutional Review Board of the University of Santo Tomas Hospital (USTH; IRB-2016-11-191-IS-A1/CR2) in Manila and the Research Ethics Review Committee of the

Mariano Marcos Memorial Hospital and Medical Center (MMMH-MC; RERC-17-001) in Batac, Ilocos Norte, Philippines. All participants gave their written informed consent.

Consent for Publication

Not applicable

Availability of Data and Materials

All data collected and analyzed in this study will be made available to the reviewers by the corresponding author upon request.

Competing Interests

The authors declare that they have no competing interests.

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AUTHOR'S CONTRIBUTIONS

The following are the contributions of the individual authors:

EMM - data collection and preliminary analysis of results, primarily responsible in peptide selection and preparation of manuscript

GAB - data collection and selection of peptides

AMC - sampling and data collection

AF - sampling and acquisition of informed consent

TSO - conceptualization of the study, sample screening

PMA - conceptualization of the project, supervision and design of the ELISA experiments and preparation of the manuscript

LAG - conceptualization of the project, supervision of epitope mapping and peptide ELISA experiments and primarily involved in preparation of manuscript

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