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DETECTION OF BACTEROIDES FRAGILIS BACTERIA IN TUMORS OF COLORECTAL CANCER PATIENTS

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ABSTRACT

Detection of Bacteroides fragilis bacteria in tumors of colorectal cancer patients

Objective: To investigate the presence of Enterotoxigenic Bacteroides fragilis (ETBF) in colorectal cancer (CRC) patients in Vietnam.

Methods: A cross-sectional descriptive study was conducted using molecular biology techniques and a convenience sampling method. The study included 123 patients diagnosed with colorectal cancer in 2023–2024 at Military Hospital 175, Vietnam.

Results: Using a range of recombinant plasmid concentrations from 106 to 1 copy alongside DNA extracted from healthy human blood samples, the PCR method demonstrated detection sensitivity down to 10 bacterial copies per clinical sample. Optimized PCR conditions were applied to the 123 CRC samples, identifying *B. fragilis* in 82 samples, corresponding to a positivity rate of 66.7%. No correlation was observed between the presence of *B. fragilis* and patient characteristics such as age, sex, tumor differentiation, tumor location, or invasion depth.

Conclusion: The positivity rate for Enterotoxigenic Bacteroides fragilis among CRC patients in Vietnam is relatively high. These findings suggest that microbiota alterations could have potential as a biomarker for early detection of colorectal cancer.

Keywords: Enterotoxigenic Bacteroides fragilis, CRC, PCR.

1. INTRODUCTION

The human gut microbiota consists of approximately 100 trillion microorganisms, playing a vital role in digestion, pathogen defense, and immune system maintenance. Thanks to immune-regulatory mechanisms, the body remains healthy despite the presence of pathogenic bacteria, as the microbiota remains balanced. However, when this balance is disrupted, the introduction or increase in harmful microorganisms leads to dysbiosis. Dysbiosis can be categorized into three types: (i) depletion of beneficial bacteria, (ii) overgrowth of harmful bacteria, and (iii) loss of microbial diversity. Such disruptions are associated with various diseases, including diabetes, obesity, neurodegenerative disorders, and cancer [1] . It has been reported that bacterial factors contribute to up to 20% of cancer cases [1]. Animal studies have demonstrated that bacteria can promote colorectal cancer (CRC) development through direct interaction with the host immune system, production of cancer-related metabolites, and release of toxins that activate oncogenes[1].

Advances in next-generation sequencing (NGS) technology and metagenomics not only provide new perspectives on exploring the gut bacterial ecosystem but also highlight the association between gut bacteria and CRC. A meta-analysis of metagenomic and metataxonomic data comparing the gut microbiota of CRC patients with healthy individuals identified several bacteria, such as Fusobacterium nucleatum, Enterotoxigenic Bacteroides fragilis (ETBF), Enterococcus faecalis, pks+E.coli, and Peptostreptococcus anaerobius, that are associated with cancer progression [2,3]. These cancer-associated bacteria have been linked to CRC across diverse populations [4,5]. *B. fragilis* is a common bacterium in the gut microbiota of healthy individuals; however, an increase in the population of toxin-producing *B. fragilis* strains is associated with the initiation and progression of CRC [6]. Global statistical reports indicate that the prevalence of this bacterium in Asian countries such as China, Japan, and Iran is approximately 60%, significantly higher than in European and American

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countries (6-37%) [2,4]. Thus, this study was conducted with the objective of investigating the presence of Enterotoxigenic Bacteroides fragilis (ETBF) in CRC patients in Vietnam.

2. SUBJECTS AND RESEARCH METHODS

2.1. Study Subjects

- Sample Collection: The study was conducted on 123 patients diagnosed with colorectal cancer (CRC) in 2023–2024 at Military Hospital 175.

- Positive CRC Samples: Three CRC samples that tested positive for Bacteroides fragilis were provided by the Biomedical Laboratory, Faculty of Biology, Hanoi University of Science.

- Primers

+ Primer Design: Primers for detecting ETBF were designed based on the toxin-producing gene sequence (Bft-1 gene).

+ Synthesis: The nucleotide sequences of the primers were synthesized by IDT (Integrated DNA Technologies) and are presented in Table 1.

Table 1. Nucleotide sequences used in this study

Primer rname	Sequence (5'-3')	Size
Bft-1-F	GTACATGTTTCTATGGATA-AGCGT	100 bp
Bft-1-R	CCAGTATAAAGCTGG-TAGAATCC	

Recombinant Plasmids Used for Standard Construction: The standards used in this study were mixtures of recombinant plasmids containing the Bft-1 insert combined with normal blood samples. The plasmids were provided by the Biomedical Laboratory, Faculty of Biology, Hanoi University of Science, Vietnam National University.

2.2. Methodology

- Study design: A descriptive cross-sectional study employing several molecular biology techniques was conducted, including the following:

+ DNA Extraction:

Total DNA was extracted from tissue samples using the Quick DNA Mini Prep Plus Kit (Zymo Research, USA) according to the manufacturer's instructions. DNA concentration and purity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

+ Determination of Plasmid Copy Number for Standards: Plasmid concentration was measured using the NanoDrop 2000 spectrophotometer (Thermo Scientific). The plasmid copy number was then calculated using the following formula:

$$\text{Plasmid Copy Number} = \frac{6.023 \times 10^{23} \times \text{Amount of DNA (g)}}{\text{DNA length (bp)} \times 650 \times 10^{9}}$$

Recombinant plasmids with copy numbers ranging from 106 to 1 copy were mixed with 50 ng of human blood DNA to generate standard samples, as outlined in Table 2.

Table 2. Proportions of Plasmid Mixtures for Standard Preparation

	Plasmid copy number/µl						
PC	PC6	PC5	PC4	PC3	PC2	PC1	PC0
p. Bft-1	106	105	104	103	102	10	1
gDNA	50ng	50ng	50ng	50ng	50ng	50ng	50ng

+ *Quantification of DNA Template for PCR Reactions:* To optimize the DNA template concentration for PCR reactions, three CRC tissue samples previously confirmed positive for ETBF were used. Total DNA was tested at a range of concentrations: 25 ng, 50 ng, 100 ng, 150 ng, and 250 ng. The PCR reactions were conducted using GoTaq Green Mastermix (Promega) with a primer concentration of 0.2 µM for each reaction.

+ *PCR Conditions: Initial Denaturation:* 95°C for 5 minutes. Denaturation: 95°C for 30 seconds

Annealing: 59°C for 30 seconds. Extension: 72°C for 30 seconds. Cycles: 40. Final Extension: 72°C for 5 minutes.

+ *PCR Detection of ETBF in CRC Samples:* PCR amplification targeting the bft-1 sequence was conducted to detect ETBF in 123 CRC tissue samples. The PCR reactions followed the optimized conditions described above.

2.3. Statistical Analysis: Statistical analyses were performed to evaluate the association between the presence of Bacteroides fragilis and the histopathological characteristics of CRC samples. Qualitative tests, including Fisher's Exact Test and Chi-square test, were applied. All statistical analyses were conducted using SPSS software version 25.0.

3. RESULTS

3.1. Sequencing Results

The primer pairs were designed based on specific genes of each bacterial strain and tested on several tissue samples. Sequencing results of the positive samples confirmed the accuracy of the designed primer pairs. The PCR products were cloned to prepare standard samples.

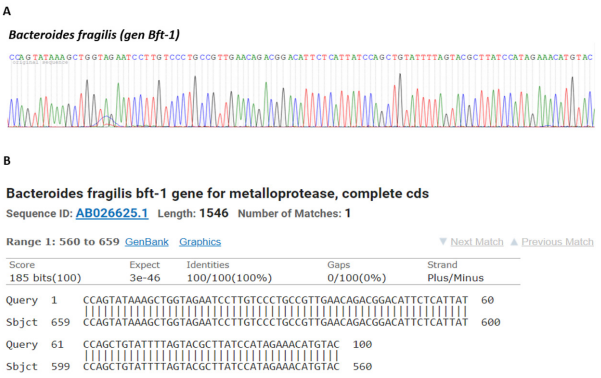

Figure 1.

(A) Sequencing results of the PCR product. (B) Sequence alignment of the 100 bp Bft-1 gene amplification with the NCBI GenBank database. "Query": Sequence of the PCR product to be compared. "Sbjct": Reference gene sequence (ID: AB026625.1). "Identities": Percentage similarity, calculated as the ratio of identical nucleotides to the total number of nucleotides compared.

3.2. Determination of DNA Template Quantity for PCR

To determine the maximum allowable DNA template concentration for specific amplification of the Bft-1 gene of Bacteroides fragilis, we tested a range of total DNA concentrations (25 ng, 50 ng, 100 ng, 150 ng, and 250 ng) using three CRC tissue samples previously confirmed positive for this bacterium.

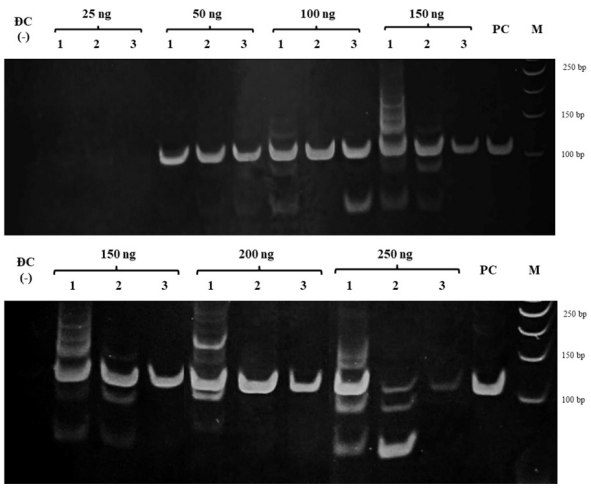

Figure 2. Electrophoresis results of PCR products testing DNA concentrations (25 ng – 250 ng) from three colorectal cancer (CRC) samples (1–3) detecting ETBF on an 8% polyacrylamide gel.

NTC: Negative control without DNA template. PC: Positive control – one of the three CRC samples previously confirmed positive for Bacteroides fragilis. M: 50 bp DNA ladder (GeneRuler).

The electrophoresis results indicate that a DNA concentration of 25 ng does not provide sufficient template for PCR amplification. With a DNA template concentration of 50 ng, a specific band of the expected size (100 bp) was observed. For DNA concentrations ranging from 100 ng to 250 ng, multiple nonspecific bands were detected in the PCR products. Thus, 50 ng is the optimal DNA template concentration for specific amplification of the bft-1 gene, enabling the detection of Bacteroides fragilis in CRC tissue samples.

3.3. Investigation of PCR Detection Limit

To determine the detection limit of the PCR reaction for amplifying Bacteroides fragilis in CRC tissue, we prepared a series of recombinant plasmid concentrations ranging from 106 to 1 copy mixed with DNA from healthy human blood. The PCR results showed that the assay could detect as few as 10 bacterial copies in the tissue samples.

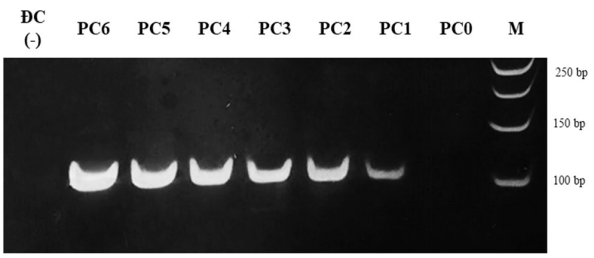

Figure 3. Electrophoresis results of PCR products testing reaction sensitivity.

3.4. PCR Results for Detecting Bacteroides fragilis in CRC Samples

Using the optimized PCR conditions, we applied the reaction to 123 CRC tissue samples. The results showed 82 samples tested positive for Bacteroides fragilis, corresponding to 66.7%.

3.5. Association Between Bacteroides fragilis and Histopathological Characteristics

Analysis of the presence of Bacteroides fragilis and histopathological characteristics of CRC patients revealed no correlation between the bacterium and age, gender, tumor differentiation grade, tumor location, or tumor invasion level (Table 5).

Table 4. Correlation between the presence of Bacteroides fragilis and histopathological characteristics

Histopathological features		*B. fragilis* infection		
		Negative	Possitive	p
Age	≤ 50	3	9	0.749[b]
	> 50	38	73	
Gender	Male	28	49	0.431[a]
	Female	13	33	

Differentiation	Highly and moderately	31	74	0.074[b]
	Poor	6	4	
	NI	8		
Degree of invasion	pT1 and pT2	6	21	0.320[a]
	pT3	17	38	
	pT4	7	9	
	NI	25		
Sample location	Colon	26	47	0.563[a]
	Rectum	15	35	

Note: a: Chi-square test; b: Fisher's exact test; NI: No information

4. DISCUSSION

The positivity rate for Bacteroides fragilis carrying the bft-1 toxin gene in colorectal cancer (CRC) patients in Vietnam was 66.7% (n=123). This rate is consistent with reports from several other Asian countries. In a study by Youlian Zhou et al., 64.37% of 97 CRC tumor samples in China tested positive for *B. fragilis* using 50 ng of DNA template [4]. Similarly, Zamani S. et al. identified a positivity rate of 47% in a study on 68 CRC samples from Iranian patients. In contrast, the positivity rate for *B. fragilis* in Australian CRC patients was significantly lower at 6.1%, as reported by Jihoon et al., who analyzed 1,697 CRC samples using 20 ng of DNA template [7].

The *B. fragilis* positivity rate in CRC patients in Vietnam aligns with findings from other Asian countries and is higher than rates reported in Europe and Africa. A 2023 global cancer statistic indicated that CRC incidence rates are highest in Oceania and Europe and lowest in Asia [8]. Interestingly, while *B. fragilis* is considered a causative agent of CRC, countries with higher *B. fragilis* infection rates tend to have lower CRC incidence, and vice versa. This paradox suggests the need for a comprehensive analysis of the gut microbiota to better understand interactions between bacterial strains and the host. Additionally, identifying beneficial bacteria that help maintain gut microbiota balance is crucial.

Our findings align with Jihoon et al., who reported no correlation between the presence of *B. fragilis* and histopathological features such as age, gender, and tumor location in 1,697 CRC tumor samples [7]. Similarly, a recent study in Vietnam by Nguyen Duy Truong et al. (2023) found a *B. fragilis* infection rate of 71.2% in 139 CRC patients. This study also concluded that *B. fragilis* is independent of factors such as age, gender, and tumor location in contributing to CRC risk [9].

However, contrasting results have been reported globally. In 2017, Purcell et al. from the University of Otago, New Zealand, found a significant correlation between *B. fragilis* and tumor location in the colon, including the left and right colon, transverse colon, sigmoid colon, and cecum ($p<0.05$) [10]. This discrepancy may be explained by the fact that our study focused on Stage II invasive tumors, and information on tumor location was not fully detailed. Furthermore, the absence of comprehensive data on tumor invasion and differentiation grades in our 123 samples limited our ability to establish a correlation between *B. fragilis* presence and CRC histopathological characteristics.

Therefore, future studies should increase the sample size and ensure detailed information on tumor invasion levels and diverse sampling locations to clarify the relationship between *B. fragilis* presence and histopathological features in CRC patients in Vietnam.

5. CONCLUSION

The positivity rate for Bacteroides fragilis was 66.7% (n = 123). The positivity rates for Fusobacterium nucleatum and Bacteroides fragilis in colorectal cancer (CRC) patients in Vietnam are higher compared to those reported in European and American populations.

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