RESEARCH ARTICLE

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Elevated Interleukin-1β and Deep Tissue Cryptosporidium Infection in Colorectal Cancer: A Novel Association

Sri W. Handayani[1,2], Nuzulia Irawati[1,3]*, Djong H. Tjong[1,4], Tofrizal Alimuddin[1,5], Avit Suchitra[6]

Abstract

Objective: This research aimed to investigate the increase in the pro-inflammatory cytokines interleukin -1β, associated with colorectal cancer and *Cryptosporidium spp*. infection. **Methods:** A total of 34 patients diagnosed with colorectal cancer were investigated at various stages. Fecal, blood, and tissue paraffin-block samples were collected from each patient. Furthermore, modified Ziehl-Neelsen staining, enzyme-linked immunosorbent assay (ELISA), and immunohistochemical staining were performed. Statistical tests were performed using the Kruskal-Wallis, Mann-Whitney, and Chi-Square tests. **Results:** *Cryptosporidium spp*. infection was found in 47.06% of patients with colorectal cancer. *Cryptosporidium spp*. spread throughout colorectal cancer tissue from the epithelium to the serous layer. Endogenous stages of *Cryptosporidium spp*. were observed within the submucosa, including its presence in the vascular submucosal region. Statistical tests showed no association between *Cryptosporidium spp*. infection with IL-1β levels ($p > 0.05$) due to heterogeneous data. **Conclusion:** This research strengthens the association between *Cryptosporidium spp*. infection and colorectal cancer, with new evidence regarding active, chronic, invasive, and potentially extraintestinal infection. However, there is a trend of increasing serum levels of IL-1β in colorectal cancer patients infected with *Cryptosporidium spp*. compared to those who are uninfected individuals. These results provide a basis for further investigation into alternative pathogenic mechanisms by which the parasite may contribute to colorectal carcinogenesis independently or with various inflammatory pathways.

Keywords: Cryptosporidium- colorectal cancer- inflammation- interleukin-1β- chronic infection- extraintestinal infection

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Introduction

Colorectal cancer is a significant global health burden due to high rates of cancer-related morbidity and mortality [1]. Approximately 10% of cases occur worldwide, accounting for 9.4% of all deaths [2]. Despite advances in screening and treatment, the exact etiology and contributing risk factors, particularly those related to infection and inflammation, have been extensively investigated. Evidence has shown that chronic infection plays an important role in colorectal carcinogenesis, but the impact is often overlooked [3]. In this context, *Cryptosporidium spp*., an intracellular obligate parasitic protozoan associated with gastrointestinal infection, has gained attention as a potential cofactor contributing to the development of colorectal cancer [4].

Cryptosporidium spp. is known to cause diarrhea in immunocompetent individuals, but results in severe and persistent gastrointestinal diseases in immunocompromised patients [5]. Recent research have shown that infection with *Cryptosporidium spp*. lead to prolonged inflammation, epithelial damage, and changes in the gut microenvironment, creating a pro-tumorigenic environment in mouse model animals [4, 6]. However, the link between *Cryptosporidium spp*. and colorectal cancer remains largely unexplored regarding the underlying mechanisms.

The pro-inflammatory cytokine interleukin-1β (IL-1β) is widely recognized as a key mediator of inflammation-induced carcinogenesis. Increased levels of IL-1β have been associated to tumor growth, angiogenesis, and metastasis in various cancers, including colorectal cancer [7-11]. In contrast, IL-1β is an essential cytokine for the host immune response and resistance to pathogens, and is

*[1]Doctoral Program in Biomedical Sciences, Faculty of Medicine, Andalas University, Padang, Indonesia. [2]Department of Health Analyst, Faculty of Pharmacy and Sciences, Muhammadiyah Prof. Dr. Hamka University, Jakarta, Indonesia. [3]Department of Parasitology, Faculty of Medicine, Andalas University, Padang, Indonesia. [4]Department of Biology, Faculty of Mathematics and Natural Sciences, Andalas University, Padang, Indonesia. [5]Department of Anatomy Pathology, Faculty of Medicine, Andalas University, Padang, Indonesia. [6]Department of Surgery, Faculty of Medicine, Andalas University, Padang, Indonesia. *For Correspondence: nuzuliairawati03@gmail.com*

produced during infection and tissue injury. An increase in IL-1β enhances tissue damage, results in systemic inflammation, and contributes to the formation of an immunosuppressive tumor microenvironment [6].

Histopathological and immunohistochemical analyses provide insight into the presence of *Cryptosporidium spp*. within colorectal tissue, supporting the potential role as a cofactor in the pathogenesis of colorectal cancer [12]. Additionally, this may be associated with elevated serum IL-1β levels. Research on *Cryptosporidium spp*. and colorectal cancer has primarily focused on prevalence, without reporting the morphological site of infection, and has shown increased levels of IL-1β. *Cryptosporidium spp*. infection is associated with increased serum IL-1β levels in patients with colorectal cancer. Therefore, this research aimed to investigate the presence of *Cryptosporidium spp*. in newly diagnosed patients with colorectal cancer and analyze the association with increased serum IL-1β levels, exploring the role of *Cryptosporidium spp*. in the induction of chronic inflammation, promoting the development of colorectal cancer. Data were analyzed on the prevalence, morphological localization of infection, and inflammatory marker IL-1β to show the clinical implications of *Cryptosporidium spp*. Understanding the relationships contributed to the development of more effective diagnostic and preventive strategies for the management of colorectal cancer.

Materials and Methods

Cross-sectional research of colorectal cancer patients diagnosed at a tertiary care hospital in Padang, Indonesia, was conducted from April 2023 to September 2023. This research was approved by the Ethics Committee of the Faculty of Medicine, Andalas University (No.137/UN.16.2/KEP-FK/2023) and the Ethics Committee of the RSUP. Dr. M. Djamil Padang (LB.02.02/5.7/213/2023). The participants were informed and provided consent forms before participating.

Participants

Eligible participants included individuals aged 28-78 years diagnosed with colorectal cancer who had not received any oncological therapy. Patients with a family history of cancer, other malignancies, or immunocompromised conditions were excluded. A total of 34 patients were recruited for the research, and data, including age, sex, cancer location, stage, and histological subtypes, were obtained from medical records.

Sample Collection

Fecal and Blood samples were collected from each participant for the detection of *Cryptosporidium spp*. and measurement of IL-1β levels. The stool samples were processed within 24 h of collection, and the serum samples were stored at -20°C for further examination. Tissue samples pinned with paraffin were obtained from the Laboratory of Anatomical Pathology at Dr. M. Djamil Padang Hospital for the detection of Cryptosporidium antigens and determination of the location of infection.

Cryptosporidium Detection

Modified Ziehl-Neelsen

The fecal samples were processed using modified Ziehl-Neelsen staining to identify Cryptosporidium oocysts. The slides were examined under a light microscope using oil at x magnification of 1000x by three trained independent observers. *Cryptosporidium spp*. were identified as 4–6micron spherical oocysts that were pink on a blue background.

Immunohistochemical

Tissue samples in paraffin were processed using immunohistochemical staining to detect antigens and sites of *Cryptosporidium spp*. using the Medikbio Monoclonal Antibody Cryptosporidium BSM-2313M, following the recommended procedure. Briefly, paraffin blocks were cut into sections of 3-4μm and affixed to poly-l-lysine-coated slides. The slides were heated on a hot plate (56 - 60 °C) for 10 minutes and left overnight in an incubator (37 C). Deparaffinization, rehydration, antigen uptake, and blocking of endogenous peroxidase activity were also performed. The primary antibody used was a monoclonal antibody against Cryptosporidium (1:100 dilution). Chromogenic reactions were performed using DAB substrates, followed by reverse staining with Mayer's hematoxylin, dehydration, and cover slipping. Furthermore, the slides were examined under a light microscope, and *Cryptosporidium spp*. appeared brown. Slide readings were performed at 400x and 1000x magnification, and measurements were taken for validation in sizes ranging from approximately 4 to 6μm. The assessment was also conducted by two pathologists in a double-anonymized manner. Assessments were carried out on all the slide view lines per sample preparation.

Measurement of IL-1β Levels

Serum levels of IL-1β were measured using enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions (BT LAB Bioassay Technology Laboratory, China: E0143Hu QLB00B ELISA kit). Meanwhile, absorbance was measured at 450 nm using a microplate reader, and the results were expressed as picograms per milliliter (pg/mL).

Statistical analysis

Data were analyzed using SPSS version 25.0 (IBM Corp., Armonk, NY, USA). Descriptive statistics were calculated for the demographic and clinical characteristics. Categorical variables were presented as frequencies and percentages, while continuous variables were reported as mean ± standard deviation. The relationship between *Cryptosporidium spp*. and serum IL-1β levels were assessed using the Mann-Whitney test. The difference in mean serum IL-1β levels at the stage was analyzed using the Kruskal-Wallis test. In addition, the relationship between *Cryptosporidium spp*., demographic, and clinical characteristics was assessed using the chi-square test with a statistical significance set at $P < 0.05$.

Results

Demographic and Clinical Characteristics

A total of 34 patients with colorectal cancer were included in this research. The average age of the patients was 51.53±13.65 years, ranging from 28 to 78 years, with 58.82% of the patients being male and 41.18% being female. Adenocarcinoma was the most commonly reported histological subtype (79.41%). Table 1 shows the demographic and clinical characteristics of the participants.

Prevalence of Cryptosporidium Infection

A total of 16 out of 34 (47.06%) colorectal cancer patients were found to have *Cryptosporidium spp*. in stool samples. The prevalence of *Cryptosporidium spp*. was equally reported in males and females (50%), with most cases occurring in individuals aged 20-40 years. *Cryptosporidium spp*. was most commonly reported in stage II and III cancers, at 43.75% and 37.50%, respectively. Meanwhile, left-sided cancer and adenocarcinomas at 87.50% and 93.75% were particularly found in the rectum. Statistical tests reported no association between age, sex, stage, cancer location, histological subtype, and *Cryptosporidium spp*. ($P > 0.05$). Table 1 shows the distribution of infection according to demographic and clinical characteristics.

Location of Cryptosporidium Infection in Cancerous Tissues

A total of 16 tissues in the paraffin block were examined, and *Cryptosporidium spp*. was reported to spread throughout colorectal cancer tissue, from the epithelium to the inner layer of the serosa, showing chronic infection. The endogenous stage of *Cryptosporidium spp*. development was found within the submucosa. *Cryptosporidium spp*. in the vascular submucosa also showed extraintestinal spread. Figure 1 reports the morphological distribution of *Cryptosporidium spp*. sites in colorectal cancer tissues.

IL-1β Levels

The mean serum IL-1β levels among all samples were 1,509.97 ± 1,053.53 pg/mL, with the lowest and highest values at 807.1 pg/mL and 6,013.8 pg/mL, respectively. There was a significant difference in IL-1β levels between stages (I, II, III, IV) ($p < 0.05$). Table 2 shows the average distribution of serum IL-1β levels according to the disease stage.

The Relationship between Cryptosporidium Infection and IL-1β Levels

Colorectal cancer patients who were positive for *Cryptosporidium spp*. had a higher average serum IL-1β level of 1,693,31±1,446.30 pg/mL than those who were negative at 1,347.01±499.01 pg/mL. However, the results of the statistical test were not significant ($p>0.05$), as there

Table 1. Distribution of Demographic and Clinical Characteristic in Cryptosporidium Infection

Characteristics	Positive n (%)	Negative n (%)	Total	P value
Total Cases	16 (47.06)	18 (52.94)	34 (100)	
Age (years)				
Mean 51,53±13,656				
Range 28-78				
20-40	5 (31.25)	1 (5.55)	6 (17.65)	0.145
40-60	8 (50.00)	12 (66.67)	20 (58.82)	
>60	3 (18.75)	5 (27.78)	8 (23.53)	
Gender				
Male	8 (50.0)	12 (66.67)	20 (58.82)	0.524
Female	8 (50.0)	6 (33.33)	14 (41.18)	
Stage TNM				
Stage I	3 (18.75)	0	3 (8.82)	0.207
Stage II	7 (43.75)	8 (44.44)	15 (44.12)	
Stage III	6 (37.50)	9 (50.00)	15 (44.12)	
Stage IV	0	1 (5.56)	1 (2.94)	
Cancer location				
Right side	2 (12.50)	7 (38.89)	9 (26.47)	0.125
Left side	14 (87.50)	11 (61.11)	25 (73.53)	
Subtypes of histology				
Adenocarcinoma	15 (93.75)	12 (66.67)	27 (79.41)	0.258
Mucinous adenocarcinoma	1 (6.25)	4 (22.22)	5 (14.71)	
Signet ring cell carcinoma	0	1 (5.55)	1 (2.94)	
Adenosquamous cell carcinoma	0	1 (5.55)	1 (2.94)	

The P value is derived from the Chi-Square Test

Table 2. Distribution of Serum IL-1B Levels by Stage

Stage	n	Average IL-1β (Mean±SD) (pg/ml)	P value
Stage I	3	1.125.457±478.230	0.040
Stage II	15	1.121.13±374.210	
Stage III	15	1.932.667±1.424.523	
Stage IV	1	2.151.400	
Total	34	1.509.971±1.053.526	

The P value is derived from the Kruskal-Wallis nonparametric test

Table 3. Average Serum IL-1B Levels by *Cryptosporidium* Infection

Cryptosporidium	n	Average IL-1β (Mean±SD) (pg/ml)	P value
Positive	16	1.693.306±1.446.299	0.523
Negative	18	1.347.006±499.099	

The P value is derived from the Mann-Witney nonparametric test

was no association between *Cryptosporidium spp*. and IL-1β levels due to heterogeneous data. Table 3 reports the average serum IL-1β levels based on *Cryptosporidium spp*.

This research showed a high prevalence of *Cryptosporidium spp*. in colorectal cancer patients, but the significance was absent when associated with levels of the pro-inflammatory cytokine IL-1β. However, there was a tendency for serum IL-1β levels to increase more in individuals infected with *Cryptosporidium spp*. positive colorectal cancer group. The site of *Cryptosporidium spp*., which was widespread up to the serous inner layer of colorectal cancer tissue, indicated a chronic, active, and invasive infection that spread to extraintestinal sites. These results provided new evidence in line with previous research on the association between *Cryptosporidium spp*. and colorectal cancer.

Discussion

This research provides important insights into the prevalence and characteristics of *Cryptosporidium spp*. in patients with CRC and explores the relationship with IL-1β levels. The results show that *Cryptosporidium spp*. is detected in almost half of the total sample of patients with CRC. This substantial prevalence is in line with several epidemiological reports showing an increased risk of infection with *Cryptosporidium spp*. in gastrointestinal cancer [13-19]. The prevalence ranges from 12.6% to 47.5% in Poland, Lebanon, Iran, Egypt, and China. Recent meta-analyses also support a positive association between *Cryptosporidium spp*. and cancer, with a significant odds ratio [20, 21]. However, this research is the first to report *Cryptosporidium spp*. in an Indonesian colorectal cancer

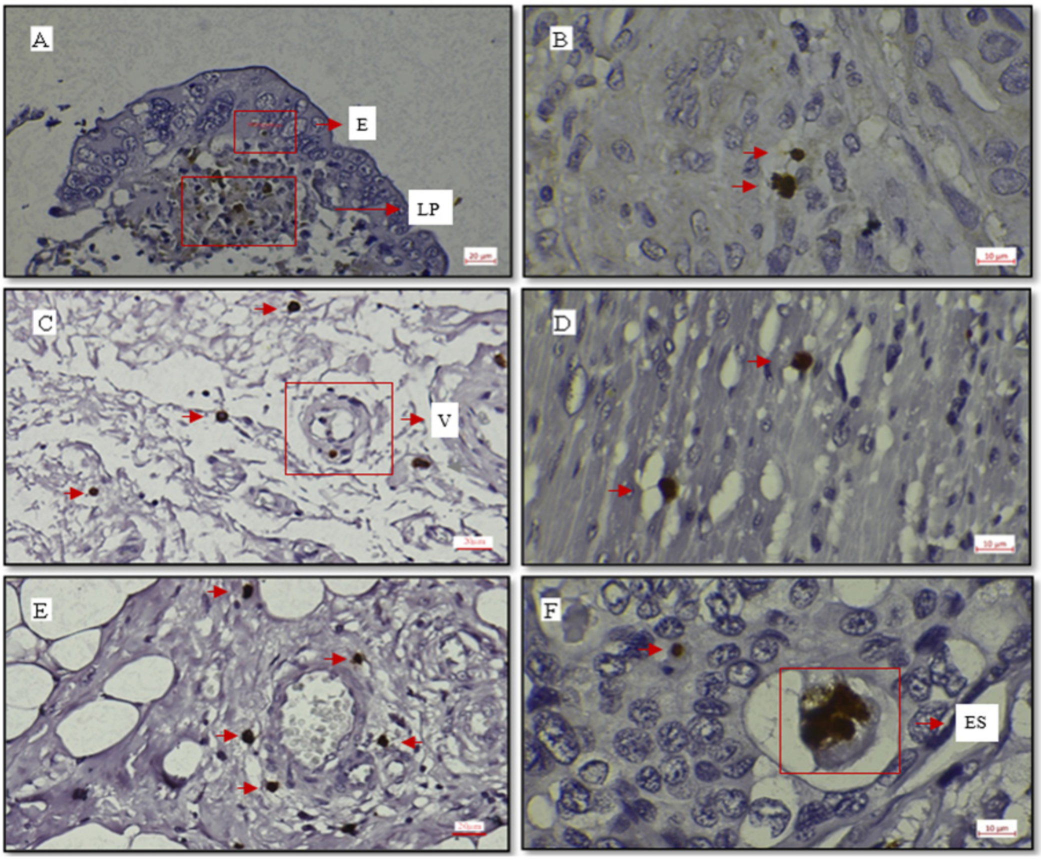

Figure 1. Immunohistochemical Micrograph photo of *Cryptosporidium spp*. in colorectal cancer tissue. *Cryptosporidium spp*. is widespread in colorectal cancer tissues, A. in the epithelial (E) and lamina propria (LP) compartments in the mucosa, B. stroma in the sub mucosa (red arrow), C. vascular (V) and sub mucosa (red arrow), D. muscularis propria in the sub serosa (red arrow), E. between the vascular and adipose tissue in the serosa (red arrow), and F. the endogenous stage of *Cryptosporidium spp*. in the sub mucosa (ES). Photographs were taken using Primostar 3 Axiocam 208 (A, B, D, F), and Olympus DP20 (C&E). Magnification: 400x, bar= 20 µm (A, C, E) and 1000x, bar= 10µm (B, D, F).

population.

The most prominent aspect of the research is the location of infection with *Cryptosporidium spp*., ranging from the epithelium to the serosa, as well as the discovery of the stage of endogenous development in the submucosa of tissue (Figure 1.F). Presence of endogenous stages of *Cryptosporidium spp*. development in the deeper layers of tissue strongly suggests an active and chronic infection rather than surface contamination. Furthermore, the discovery of *Cryptosporidium spp*. in the vascular submucosa of colorectal cancer tissue (Figure 1.C) shows the spread of infection to extraintestinal sites. Infection can spread to all digestive and extraintestinal organs through the bloodstream and originate from the colon [22].

Cryptosporidium spp. are known to cause diarrhea confined to the intestinal epithelium and extraintestinal spread, particularly in immunocompromised individuals [23] and mice [24]. The results in colorectal cancer patients, who often experience degrees of immunosuppression due to the disease, support the possibility of the systemic spread and show the broader potential pathogenicity in the context of malignancy.

The implications of infection with *Cryptosporidium spp*. active, chronic, invasive, and potentially spreading extraintestinally in colorectal cancer are significant. Parasitic infection triggers epithelial cell damage, tight junction disorders, and persistent chronic inflammatory responses [6,25-27]. This chronic inflammatory environment is a well-established risk factor for cancer development and promotes cell proliferation, angiogenesis, and metastasis [9, 28, 29]. Some proposed mechanisms linking *Cryptosporidium spp*. to carcinogenesis involve the manipulation of cellular signaling pathways, including PI3K, NF-κB, Wnt, and p38/MAPK. These pathways contribute to tumor cell survival and the development of tumor-supportive microenvironments. Virulence factors of *Cryptosporidium spp*., such as ROP1, sPLA2, and miRNAs, also disrupt host cell stability and alter the expression of cancer-relevant genes, worsening inflammation and tissue damage [30].

Even though *Cryptosporidium spp*. Infection is widely known to trigger an inflammatory response; however, no statistically significant association has been reported between *Cryptosporidium spp*. and IL-1β levels in patients with CRC. The Mann-Whitney nonparametric statistical test obtained a p-value of 0.523 ($p > 0.05$), showing the absence of a significant relationship. However, serum IL-1β levels were higher in the group that tested positive for *Cryptosporidium spp*. This difference was not statistically significant due to the relatively small sample size ($n = 34$) and the inhomogeneous nature of the IL-1β level data. The results suggest an interesting biological trend that warrants further investigation using a larger sample size to achieve sufficient statistical power.

Increased levels of IL-1β in colorectal cancer are associated with clinicopathological features, where IL-1β is overexpressed in epithelial and metastatic colorectal cancers. Meanwhile, increased levels of IL-1β are associated with improved growth and invasion of colorectal cancer [31]. An increase in the serum levels is associated with infection by *Cryptosporidium spp*. but this has not been well-documented. This research shows very high levels of IL-1β (6,013.8 pg/ml) in colorectal cancer patients who are positively infected with *Cryptosporidium spp*. Colonoscopic examination reports that the tumor spreads to the intrahepatic, intraperitoneal, and distal vaginal rectum. These results suggest that chronic infection caused by *Cryptosporidium spp*. may contribute to increased IL-1β levels and disease severity, requiring further research to explore the relationship.

Infection of epithelial cells with *Cryptosporidium spp*. increases cell permeability and causes loss of IL-1β-induced tight junction (TJ) function. This loss leads to translocation of luminal populations into the cytoplasm, contributing to inflammatory processes in the intestine. The regulatory signaling pathway mediates the increased permeability of IL-1β-induced intestinal TJ, activation of nuclear transcription factor kappa factor (NFκB) and myosin light-chain kinase gene, as well as modulation of the post-transcriptional okludine gene by microRNAs [27]. *Cryptosporidium spp*. spreads throughout the structure of colorectal cancer tissue to the inner layer of the serosa, reporting massive intestinal leakage with the ability to trigger systemic inflammation. Induced systemic inflammation *Cryptosporidium spp*. contributes to the formation of an immunosuppressive tumor microenvironment [6].

In the tumor microenvironment, IL-1β is sourced from active monocytes, macrophages, and myeloid cells [32]. Upregulation of IL-1β in colorectal cancer regulates epithelial-mesenchymal transition, cell invasion and migration, proliferation, and clone formation through the induction of autophagy [29]. Furthermore, the accumulation of inflammatory cells and cytokines in the tumor microenvironment promotes the proliferation of malignant cells, metastasis, and epithelial-mesenchymal transition, leading to a loss of the innate immune response [33]. Cancer-related inflammation affects the entire body, allowing systemic cytokine levels to reflect the microenvironmental processes of the tumor [32].

Interleukin-1β, in the early stages of carcinogenesis, shows pro-inflammatory activity, increases tumor invasion, and becomes immunosuppressive [34, 35]. Research proposes that M2 macrophages are the primary source of IL-1β in patients with colorectal cancer. This is because the macrophages have been found to comprise up to half of the tumor mass in advanced stages [36, 37]. The results show that increased IL-1β levels are associated with cancer progression [38]. This research focuses on a single inflammatory marker, IL-1β, while other pro-inflammatory cytokines, such as TNF-α and IL-6, may also play a significant role in parasite-induced carcinogenesis [39]. Chronic inflammation caused by an undiagnosed infection increases the risk of cancer, and approximately 25% of tumors develop from chronic inflammation [40].

Therefore, there is no direct association between *Cryptosporidium spp*. and IL-1β levels. In this context, IL-1β remains a relevant inflammatory biomarker in colorectal cancer. Several possible explanations account for the lack of a statistically significant relationship between *Cryptosporidium spp*. and IL-1β observed.

First, there may be other pro-inflammatory cytokines or more dominant inflammatory pathways triggered by *Cryptosporidium spp*. in colorectal cancer. For example, TNF-α, IL-6, and specific chemokines play central roles in the process. Second, the immune response in cancer patients is modified or dysregulated. Therefore, the IL-1β response is not as pronounced in immunocompetent patients. Third, interactions between *Cryptosporidium spp*. and host cells in complex tumor environments modulate IL-1β production without a measurable significant increase. Further research is needed to identify the specific cytokine profiles and inflammatory pathways activated by *Cryptosporidium spp*. deep tissue infection in colorectal cancer at larger sample counts and including control populations (non-CRC).

This research recommends the inclusion of *Cryptosporidium spp*. as a companion for fecal occult blood tests (FOBT) for the early detection of parasites in patients with colorectal cancer. Examination of *Cryptosporidium spp*. is included in histopathological and immunohistochemical examinations in patients with poor prognosis and extraintestinal expansion. Therefore, these results have clinical implications in cancer diagnosis and prevention. The results strengthen the association between *Cryptosporidium spp*. and colorectal cancer, by providing strong evidence of active, chronic, invasive, and potentially extraintestinal infection. Even though a direct relationship with IL-1β is not shown to be significant, the results open opportunity for further investigation of the alternative pathogenic mechanisms contributing to colorectal carcinogenesis. Future research should focus on identifying cytokines and other relevant signaling pathways, as well as exploring the impact of *Cryptosporidium spp*. elimination of colorectal cancer progression.

Author Contribution Statement

SWH: conception and design, performing calculations, investigating research, and supervising the results of this work; NI: conception and design, data analysis; DHT: conception and design, data analysis; TA: conception and design, data analysis. All the writers discussed the results and contributed to the development of the script. All authors have read and approved the final manuscript and agree to be responsible for all aspects of the work to ensure that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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General

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Ethical Declaration

This research was approved by the Research Ethics Committee of the Faculty of Medicine, Andalas University (No.137/UN.16.2/KEP-FK/2023) and the Research Ethics Committee of Dr. M. Djamil Hospital (No. LB.02.02/5.7/213/2023).

Conflict of Interest

The authors declare no conflicts of interest

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