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High prevalence of *Cryptosporidium* infection in Iranian patients suffering from colorectal cancer

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ABSTRACT

The present investigation was designed to study the prevalence of cryptosporidiosis in the colorectal cancer patients compared to the healthy subjects. The present descriptive case-control study was performed on 174 subjects including 87 healthy people and 87 patients with colorectal cancer attending to general hospitals in Lorestan Province, Western Iran, during October 2019–August 2020. A fresh stool specimen was collected from each subject in a sterile labeled container. The collected stool samples were concentrated using the sucrose flotation method and then prepared for Ziehl-Neelsen staining for microscopic examination. All samples were also tested using the Nested-PCR assays by amplifying the 18S rRNA gene for the presence of *Cryptosporidium* DNA. Demographic and possible risk factors such as age, gender, residence, agriculture activity, history of contact with livestock, consumption unwashed fruits/vegetables, and hand washing before eating were investigated in all the studied subjects using a questionnaire. Of the 87 patients with colorectal cancer, 37 (42.5%) had *Cryptosporidium* infection. A significant difference ($p < 0.001$) in the prevalence of *Cryptosporidium* spp. infections among the participants in the case and control (11, 12.6%) groups was observed. We found that cryptosporidiosis was not linked with age, gender, hand washing, agriculture activity, and history of contact with livestock in the colorectal patients. However, residence in urban areas was significantly associated with the prevalence of cryptosporidiosis. The 18 s rRNA gene of *Cryptosporidium* in 48 samples was successfully amplified by the Nested-PCR. Based on the obtained findings, *Cryptosporidium* spp. infections were observed significantly more frequently in the patients with colorectal cancer in comparison with the healthy individuals. It is suggested to carry out similar studies in various parts of Iran with larger sample sizes and further parasitological tests.

1. Introduction

Colorectal cancer is one of the most common cancers in the gastrointestinal tract. In 2020, colorectal cancer was responsible for 10.0% of worldwide cancer incidence and 9.4% of cancer deaths; whereas it is predicted that by 2040, >3.2 million new cases of the

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disease will be reported (Xi and Xu, 2021). Although the main factors involved in the development of colorectal cancer are obesity, low-vegetable and low-fruit diet, sedentary lifestyle and smoking, but the role of infectious agents cannot be overlooked (Haraldsdottir et al., 2014).

Nowadays, the effect of microbial pathogens on the development of cancers has been proven in previous studies. *Helicobacter pylori*, *Mycobacterium tuberculosis*, human papilloma virus, hepatitis B and C viruses, Epstein-Barr virus, human immunodeficiency virus and herpes virus all have proven effects on the carcinogenicity of tissues or organs, in which they are located (Bouvard et al., 2009; De Martel and Franceschi, 2009). Among the parasitic pathogens, *Schistosoma haematobium* is a blood parasitic trematode that can cause bladder cancer; in addition, some liver and bile duct trematodes such as *Opisthorchis viverrini* and *Clonorchis sinensis* are sometimes linked to cholangiocarcinoma (Oliveira, 2014; Hamid, 2018; Yang et al., 2018).

Cryptosporidium spp. are intracellular protozoan parasites with in oocysts size of 3–5 μm containing four sporozoites in the environment or in the stools of infected individuals (Ryan et al., 2014). *Cryptosporidium* spp. infect a broad spectrum of animals such as mammals, birds, amphibians, fishes and reptiles (Ryan and Hijjawi, 2015). From the 44 reported species with >120 valid genotypes of *Cryptosporidium* (Ryan et al., 2021), >21 species and genotypes have been reported in human that can cause mild to moderate gastrointestinal symptoms (Ryan and Hijjawi, 2015; Ahmed and Karanis, 2020; Mahmoudi et al., 2017). Among these species, *C. parvum* and *C. hominis* are well-known as the most prevalent etiological agents accountable for this disease in humans around the word (Ryan et al., 2014). Human is generally infected with *Cryptosporidium*, through the direct contact with infected persons or animals, or indirectly, by ingestion of contaminated food or water with oocysts (Pumipuntu and Piratae, 2018).

The prevalence of this protozoan parasite is estimated between 1 and 3.0% in advanced countries of Europe and North America, about 5.0% in Asia and 10.0% in Africa (Pumipuntu and Piratae, 2018). *Cryptosporidium* also causes diarrhea in 10.0% to 20.0% of people with acquired immunodeficiency syndrome (AIDS) in industrialized countries, and this rate is up to 50.0% in developing countries (Gerace et al., 2019). Unlike the self-limiting nature of the infection in immunocompetent people, the disease has a severe and dangerous course in people with immunodeficiency (Ryan and Hijjawi, 2015; Mahmoudi et al., 2017). Reviews have shown that cryptosporidiosis is the second cause of diarrhea-associated mortality in children lower than 5 years of age, responsible for 604,000 deaths in 2015 (Kotloff et al., 2013; Butkeviciute et al., 2021; Ryan et al., 2018).

Recently, studies have proposed that *Cryptosporidium* spp. parasites can cause malignant cancers in the gastrointestinal tract and biliary epithelium of mice with severe combined immunodeficiency (Certad et al., 2010). Most of the evidence gathered from the studies has involved human subjects so far points out towards a positive association between the presence of the *Cryptosporidium* spp. infections and an increased risk of developing colorectal cancer (Sawant et al., 2020; Kalantari et al., 2020; Sulżyc-Bielicka et al., 2012, 2018). For example, Sulzyc-Bielicka et al. (2007) have reported that among 55 patients with diagnosed colorectal cancer, *Cryptosporidium* spp. parasites were significantly found in 23 persons (43.5%). Osman et al. (2017) also have reported that the high association of *Cryptosporidium* spp. infection with colon adenocarcinoma in Lebanese patients; so that among 218 patients, the presence of *Cryptosporidium* was surprisingly reported in 21% of patients. Considering the prevalence of *Cryptosporidium* infection, reviews have reported that the rate of cryptosporidiosis among children, healthy people, and gastroenteritis and immunocompromised patients in Iran was estimated 3.6, 2.9, 1.3, and 4.5%, respectively (Berahmat et al., 2017). Based on the prevalence of this parasitic protozoan in Iran and what has been mentioned so far, the purpose of the current investigation was to study the prevalence and associated risk factors of *Cryptosporidium* infection in Iranian patients suffering from colorectal cancer.

2. Materials and methods

2.1. Ethical statement

This study was reviewed and approved by Ethical Committee of Lorestan University of Medical Sciences, Khorramabad, Iran (IR.LUMS.REC.1397.079). The patients provided their written informed consent to participate in this study.

2.2. Study subjects

The present descriptive case-control study was performed on 87 patients with colorectal cancer (before beginning oncological treatment) including 76 (87.4%) patients with colon cancer and 11 (12.6%) patients with rectum cancer, attending general hospitals in Lorestan Province, Western Iran, during October 2017–August 2018. Among the colorectal cancer patients, most of them (69 patients, 79.3%) were in stage II, with 16 patients (18.4%) in stage III and two patients (14.9%) in stage I. It should be mentioned that the

Table 1
Frequency of the diagnostic methods used for the detection of colorectal cancer.

Diagnostic methods	Test application
Colonoscopy	Early and late diagnosis
Blood tests	Evaluation tumor markers (carcinoembryonic Antigen)
Histopathological tests	Evaluation of staging of tumor
Stool test	Fecal occult blood test
Immunochemical test	Identification of prognostic markers in cancer
Computed tomography	To check the metastatic status of the tumor

staging of colorectal cancer in patients recruited in this study was based on the American Joint Committee on Cancer (AJCC) TNM system (Rosen and Sapra, 2022). The sample size was calculated based on the alpha error of 5.0%, power of 80.0% and chances of exposure in the case group of 21.0% and chances of exposure in the control group equivalent to 6% based on previous studies (Izadi et al., 2012). Sample size was estimated as two ratios calculation formulae with considering the ratio of case to control one by one. The determination of colorectal cancer was approved by an experienced gastroenterologist based on various examinations (Table 1). Moreover, healthy individuals (87 subjects) with no present or earlier gastrointestinal manifestations and patients with a negative result in colonoscopy and/or fecal blood test referred to hospitals during the above study period were included in the control group. The exclusion criteria were being disagreed to participate in the study, having previously undergone chemotherapy, having taken systemic antibiotics in the last three months and being immunocompromised.

2.3. Questionnaire

All demographic and possible risk factors such as age, gender, residence, agriculture activity, history of contact with livestock, consumption unwashed fruits/vegetables, and hand washing before eating were investigated in all the studied subjects using a questionnaire.

2.4. Collection and processing of samples

A fresh stool specimen was collected from each subject in a sterile labeled container. The collected stool samples were then transferred to the Parasitology Laboratory of the Faculty of Allied Medicine, Lorestan University of Medical Sciences, Khorramabad, Iran, for microscopic and molecular examinations. In this study, an expert technician concentrated the collected stool specimens using the sucrose flotation method, also called "Sheather's technique" (with a definite gravity of 1.21) based on the previous studies (Mahmoudvand et al., 2019).

2.5. Prevalence of Cryptosporidium by conventional microscopy

The smears provided from the case and control participants samples were stained using the modified Ziehl Neelsen assay, air dried and fixed by ethanol. Then, alkaline fushin was poured on the slides and heated until it turned to steam, but was not boiled. After 5 min, the slides were washed by water and decolorized by 2.5% sulfuric acid for 1 min, depending on the film thickness. Subsequently, the slides were counter stained with 1.0% methylene blue for 1 min and then washed, air dried and examined with the 100× objective. Finally, the samples were studied using a light microscope at 40× and confirmed at 100× to find *Cryptosporidium* oocysts (Henriksen and Pohlenz, 1981). All stool specimens were kept in 2.5% potassium dichromate at 4 °C for DNA extraction.

2.6. Prevalence of Cryptosporidium by PCR

DNA extraction of case and control specimens was carried out by means of QIAamp® Fast DNA Stool Mini Kit 50 (Qiagen, Hilden, Germany) according to the manufacturer's protocol. For DNA extraction, 0.2 g of stool samples were eluted with 100 µl elution buffer. The obtained DNA was kept in −20 °C until using. Nested-PCR was used to amplify a fragment of the 18S rRNA gene with length of produced fragments of 1325 bp and 830 bp, respectively. The primers used were: Forward (F1): 5′-TTCTAGAGCTAATACATGCG- 3′ and Reverse (R1): 5′-CCCATTTCCTTCGAAACAGGA3 3′ for the first step; Forward (F2): 5′-GGAAGGGTTGTATTTATTAGATAAAG- 3′ and Reverse (R2): 5′ -CTCATAAGGTGCTGAAGGAGTA- 3′ (Jiang et al., 2005). For the initial round of nested PCR, 100 µl amplification reaction included 2 µl template DNA, 2 µl primers (1 µM), 16 µl $MgCl_2$ (25 nM), 2 µl dNTP, 10 µl Taq buffer, and 0,5 µl Taq DNA Polymerase (Thermo Fisher Scientific, Waltham, US). In the second round of nested PCR, 4 µl PCR product as template DNA and 4 µl $MgCl_2$ (25 nM) was used as different from the first step. The cycling conditions for both round were 3 min initial denaturation step at 94 °C, followed by 35 cycles of 45 s at 94 °C, 45 s at 55 °C, and 1 min at 72 °C, and a final extension of 7 min at 72 °C. The PCR product was transferred to a 1.5% agarose gel, electrophoresed for 1 h and then stained with ethidium bromide and then visualized under UV light using Gel Doc device (Uvidoc, Gel Documentation System, Cambridge, UK).

2.7. Statistical analysis

The statistical analysis of the results was performed using the SPSS 24.0 software (SPSS Inc., Chicago, IL, USA). Chi-square tests used to examine the difference in distribution of participants among case and control groups as well as the associations between infection and studied factors. Since the colorectal cancer is more common in people over 60 years old, we classified the participants by age of <60 and ≥ 60 years. Variables significantly related with *Cryptosporidium* prevalence were analyzed as possible risk factors by means of univariate logistic regression. In order to determine the chance of exposure to *Cryptosporidium* parasite in patients with colorectal cancer compared to healthy subjects, the odds ratio with a confidence level of 95% was used. *P* values of <0.05 for associations were considered to indicate statistical significance.

3. Results

3.1. Participants

In the present case-control investigation, totally, 174 participants including 87 patients with colorectal cancer and 87 healthy individuals referred to general hospitals of Lorestan Province, Iran, were studied to evaluate the prevalence of *Cryptosporidium* spp. infections (Table 2). The mean age of the participants in the case and control groups was 59.6 ± 10.8 and 54.3 ± 7.3 years, respectively. The majority of participants were male in the case (51, 58.6%) and control (47, 54%) groups. In terms of residence, 58 (66.7%) and 69 (79.3%) participants in the case and control groups lived in urban areas, respectively, and the rest lived in rural parts. Among the participants in the case and control groups, 80 (92%) and 73 (83.9%) participants performed hand washing before eating, respectively. Moreover, 52 (59.8%) and 20 (23%) participants in the case and control groups had agriculture activities, respectively. This difference was statistically significant between the groups ($p < 0.0001$). With regard to the history of contact with livestock, 33 (37.9%) and 18 (20.7%) participants in the case and control groups had the history of livestock, respectively. This difference was statistically significant between the groups ($p = 0.012$) (Table 2).

3.2. Prevalence of Cryptosporidium spp. infections

By microscopic and PCR tests, of the 87 patients with colorectal cancer, 37 (42.5%) had *Cryptosporidium* infection. However, of the 87 healthy participants in the control group, 11 (12.6%) had *Cryptosporidium* spp. infections (Table 3). This revealed a significant difference ($p < 0.001$) in the prevalence of *Cryptosporidium* spp. infections among the participants in the case and control groups.

3.3. Statistical analysis for risk factors and logistic regression

The results also showed that the chance of exposure to *Cryptosporidium* in the patients with colorectal cancer was 5.11-fold higher than that in the control group (OR = 5.11 with the confidence interval between 2.38 and 10.95) (Table 4).

In the study of age-related subgroups, there was no significant association of between the prevalence of *Cryptosporidium* spp. infections and participants' age ($p = 0.798$). Similarly, there was no significant association between gender and the prevalence of *Cryptosporidium* ($p = 0.309$). In terms of residence, there was a significant relationship ($p = 0.032$) between the place of residence and the prevalence of *Cryptosporidium*; accordingly, 58.6% of participants living in urban areas in the case group were found positive for *Cryptosporidium*. With regard to the history of contact with livestock, there was no significant association between having a history of livestock and the prevalence of *Cryptosporidium* ($p = 0.076$). Accordingly, 54.5% (18/33) and 16.6% (3/18) participants with the history of contact with livestock in the case and control groups were found positive for *Cryptosporidium,* respectively.

Furthermore, there was no significant association between the prevalence of *Cryptosporidium* and having agriculture activities ($p = 0.404$), consumption of unwashed fruits/vegetables ($p = 0.612$), hand washing ($p = 0.985$) in the case and control groups. In multiple logistic regression, the results demonstrated that among all risk factors living in urban regions (crude OR = 2.69, 95%CI: 1.07–6.72, $P = 0.032$) was independent risk factor for *Cryptosporidium* spp. infections (Tables 5).

Table 2
Comparison of frequency distribution of epidemiological history in patients with colorectal cancer and control group.

Variable	Group		*P* value
	Case (colorectal cancer patients) No. (%)	Control (healthy individuals) No. (%)	
Age			
<60 yrs.	44 (50.6)	54 (62.1)	0.104
≥60 yrs.	43 (49.4)	33 (37.9)	
Gender			
Male	51 (58.6)	47 (54)	0.571
Female	36 (41.4)	40 (46)	
Residence			
Urban	58 (66.7)	69 (79.3)	0.06
Rural	29 (33.3)	18 (20.7)	
Agriculture activity			
Yes	52 (59.8)	20 (23)	<0.001*
No	35 (40.2)	67 (77)	
History of contact with livestock			
Yes	33 (37.9)	18 (20.7)	0.012*
No	54 (60.1)	69 (79.3)	
Consumption unwashed fruits/vegetables			
Yes	38 (43.7)	13 (14.9)	<0.001*
No	49 (56.3)	74 (85.1)	
Hand washing before eating			
Yes	80 (92)	73 (83.9)	0.103
No	7 (8)	14 (16.9)	

Table 3
Comparison the prevalence of cryptosporidiosis in the case and control groups.

Group	Diagnostic test				OR	CI (OR)	P value
	Microscopy		PCR				
	Positive No (%)	Negative No (%)	Positive No (%)	Negative No (%)			
Case (colorectal cancer patients)	32 (36.8)	55 (63.2)	37 (42.5)	50 (57.5)	5.11	.38–10.95	<0.001*
Control (healthy individuals)	9 (10.3)	78 (89.7)	11 (12.6)	76 (87.4)	-	-	-

Table 4
Frequency of *Cryptosporidium* infection in healthy people of the control group based on the demographic characteristics and associated risk factors.

Group	Microscopic test		Crude OR	95%CI	P value
	Positive No. (%)	Negative No. (%)			
Age					
<60 yrs.	6 (11.1)	49 (88.9)	1.63	0.16–4.85	0.823
≥60 yrs	5 (15.1)	28 (84.9)	1	1	1
Gender					
Male	6 (12.8)	41 (87.2)	1.07	0.13 -4.55	0.964
Female	5 (12.5)	35 (87.5)	1	1	1
Residence					
Rural	8 (11.6)	61 (88.4)		1	1
Urban	3 (16.6)	15 (83.4)	1.39	0.56–3.73	0.424
Agriculture activity					
Yes	3 (15.0)	17 (85.0)	1.52	0.21–4.48	0.778
No	8 (11.9)	59 (88.1)	1	1	1
History of contact with livestock					
Yes	2 (11.1)	15 (88.9)	1.29	0.31–4.12	0.831
No	9 (13.4)	61 (86.6)	1	1	1
Consumption of unwashed fruits/vegetables					
Yes	2 (15.4)	11 (84.6)	1	1	1
No	9 (12.6)	65 (87.4)	1.01	0.12–4.26	0.905
Hand washing before eating					
Yes	10 (12.5)	70 (87.5)	1	1	1
No	1 (14.3)	6 (85.7)	1.33	0.52–3.61	0.361

Table 5
Frequency of *Cryptosporidium* infection in patients with colorectal cancer based on the demographic characteristics and associated risk factors.

Group	Microscopic test	Crude OR		95%CI	P value
	Positive No. (%)	Negative No. (%)			
Age					
<60 yrs.	19 (43.2)	25 (56.8)	1.83	0.12–4.87	0.798
≥60 yrs	18 (41.8)	25 (58.2)	1	1	1
Gender					
Male	24 (47.1)	27 (52.9)	1.92	0.09 -4.65	0.309
Female	13 (36.1)	23 (63.9)	1	1	1
Residence					
Rural	20 (34.5)	38 (65.5)	1	1	1
Urban	17 (58.6)	12 (41.4)	2.69	1.07–6.72	0.032*
Agriculture activity					
Yes	24 (46.2)	28 (53.8)	1.45	0.6–3.48	0.404
No	13 (37.1)	22 (62.88)	1	1	1
History of contact with livestock					
Yes	18 (54.5)	15 (45.5)	2.23	0.61–5.31	0.076
No	19 (35.2)	35 (64.8)	1	1	1
Consumption of unwashed fruits/vegetables					
Yes	15 (39.5)	23 (60.5)	1	1	1
No	22 (44.9)	27 (55.1)	1.24	0.52–2.95	0.612
Hand washing before eating					
Yes	34 (42.5)	46 (57.5)	1	1	1
No	3 (42.8)	4 (57.2)	1.01	0.21–4.83	0.985

3.4. Molecular diagnosis

All samples were also tested using the nested-PCR assays for the presence of *Cryptosporidium* DNA. The 18 s rRNA gene of *Cryptosporidium* was successfully amplified in 48 samples by the Nested-PCR. However, as no Sanger sequencing analyses were conducted, so the exact *Cryptosporidium* spp. involved in the infections were unknown.

4. Discussion

The findings of the present study revealed that there was a significant difference ($p < 0.001$) in the prevalence of *Cryptosporidium* spp. infections among the participants in the case and control groups. Moreover, all samples were also tested using the Nested-PCR assays for the presence of *Cryptosporidium* DNA. The 18 s rRNA gene of *Cryptosporidium* was successfully amplified in 48 samples by the Nested-PCR.

Several human studies have demonstrated a positive association between the presence of the *Cryptosporidium* spp. infections and an increased risk of developing colorectal cancer (Certad et al., 2007; Sawant et al., 2020; Zhang et al., 2020). In a study conducted by Sulżyc-Bielicka et al. (2018) on 108 colorectal patients in Poland, the prevalence of *Cryptosporidium* spp. infections was significantly higher in colorectal patients compared with healthy individuals; in this regard, 14 (13%) colorectal patients and 5 (4%) healthy individuals were found positive for *Cryptosporidium* spp. In another study, Osman et al. (2017) demonstrated that among 218 digestive biopsies in Tripoli, Lebanon, the presence of *Cryptosporidium* spp. was significantly identified in 21% of patients with colonic neoplasia/adenocarcinoma; while, this rate was 7% *in* patients without colon neoplasia. They also reported that compared to normal biopsies, the risk of *cryptosporidiosis* considerably increased to nearly 11-fold in patients with colon adenocarcinoma. Sulzyc-Bielicka et al. (2007) reported that the frequency of *Cryptosporidium* spp. infections in colorectal cancer patients with or without diarrhea was 43.5% and 18%, respectively.

Although the precise mechanism of cancer development by *Cryptosporidium* spp. is not yet exactly clear, some studies have suggested some possible mechanisms. For example, Benamrouz et al. (2012) showed that the Wnt signaling pathway, and particularly the cytoskeleton network, was likely to be involved in the progression of *C. parvum*-induced neoplastic development and the cell migration of transformed cells. However, more investigations are required to reveal accurate mechanisms for development of this parasite.

Here, we found a significant correlation between place of residence and the prevalence of *Cryptosporidium* spp. infections. It has been previously proven that overcrowded living conditions specially in urban regions is considered as a risk factor for *Cryptosporidium* infection (Bouzid et al., 2018). Similarly, Sulżyc-Bielicka et al. (2018) showed that in patients with colorectal cancer, no significant association was observed among *Cryptosporidium* spp. infections, gender and age. Reviews also reported that the key risk factors of cryptosporidiosis were related to drinking contaminated water, having contact with infected humans or animals, ingesting contaminated food/water, and travelling to disease endemic areas (Sulżyc-Bielicka et al., 2018; Izadi et al., 2012).

In this study, there are limitations such as low sample size that can affect the results, so we suggested the larger studies with a higher sample size to obtain more accurate results in the region. A limitation of the study is multiple stool specimen's collection at 2–3 day intervals, which can increase the sensitivity of conventional microscopy. Another drawback of this study is the lack of precise identification of *Cryptosporidium* spp. to understand the predominant species in this region (although it is likely to be *C. parvum*), which must be done with more accurate molecular tests as well as sequencing tests.

5. Conclusion

Based on the obtained findings, *Cryptosporidium* spp. infections were observed significantly more frequently in the patients with colorectal cancer in comparison with the healthy individuals. Moreover, we found that cryptosporidiosis was not linked with age, gender, hand washing, agriculture activity and history of contact with livestock in the colorectal patients. However, residence in urban areas was significantly associated with the prevalence of cryptosporidiosis. Our results suggested that physicians should pay special attention to the presence of this parasite in people with colorectal cancer and prevent serious symptoms in these people by timely diagnosis and treatment.

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Availability of data and materials

Authors can confirm all relevant data are included in the article and materials are available on request from the authors.

Consent for publication

Not applicable.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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