original article

# *Cryptosporidium spp.* during chemotherapy: a cross-sectional study of 94 patients with malignant solid tumor

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**BACKGROUND:** *Cryptosporidium spp.* is a protozoan parasite that infects many vertebrate animals, including humans. Since *Cryptosporidium spp.* can cause chronic life-threatening diarrhea and severe malabsorption in immunocompromised patients, we investigated the prevalence of this parasite among patients undergoing chemotherapy for malignant solid tumors.

**OBJECTIVE:** Investigate the prevalence of *Cryptosporidium spp.* in stool samples.

**DESIGN:** Cross-sectional.

**SETTING:** Tertiary care.

**PATIENTS AND METHODS:** Stool samples were collected from adult patients with malignant solid tumors receiving chemotherapy and diarrhea. *Cryptosporidium spp.* prevalence was determined using Ziehl–Neelsen staining, ELISA, and real-time PCR targeting of the *COWP* gene.

**MAIN OUTCOME MEASURE:** The prevalence of *Cryptosporidium spp.* in patients undergoing chemotherapy for malignant solid tumors.

**SAMPLE SIZE:** 94

**RESULTS:** The prevalence was 2.1% (2/94), 5.3% (5/94), and 5.3% (5/94) as detected by Ziehl–Neelsen staining, real-time PCR and ELISA, respectively. The prevalence reached 8.5% (8/94) using all results obtained from the three methods. Among eight positive stool samples, four were positive by at least two different methods (Ziehl–Neelsen staining-ELISA or ELISA-real-time PCR) whereas the remaining four were positive by either ELISA or real-time PCR.

**CONCLUSION:** These findings show the risk of cryptosporidiosis in cancer patients and the necessity to use at least two diagnostic methods during the diagnosis of cryptosporidiosis to reach more accurate and trustworthy results.

**LIMITATIONS:** Further studies with a larger sample size are recommended.

**CONFLICT OF INTEREST:** None.

Cryptosporidiosis is a zoonotic disease caused by *Cryptosporidium spp.*, a protozoan parasite that infects the gastrointestinal tract of many vertebrate animals, including humans. Transmission of *Cryptosporidium spp.* occurs by ingesting viable oocysts through water or food contaminated with feces. Drinking, in particular, is a significant source for transmission of *Cryptosporidium* and thus, authorities in the United Kingdom and the United States have advised immunocompromised patients to boil their drinking water to prevent *Cryptosporidium spp.* transmission.[1] In immunocompromised patients such as HIV-infected, cancer or solid-organ transplant recipients, chronic life-threatening diarrhea and severe malabsorption can occur due to *Cryptosporidium spp.* whereas in healthy individuals, cryptosporidiosis causes gastrointestinal symptoms that resolve spontaneously.[2,3]

*C hominis* and *C parvum* are the two most frequently detected *Cryptosporidium* species in humans and are responsible for approximately 90% of human cryptosporidiosis cases. To date, *C hominis* has only been detected in humans while *C parvum* has been detected in many animals as well as humans.[4] In addition to these two species, *C meleagridis, C felis, C canis, C ubiquitum, C cuniculus, C viatorum, C muris, C suis, C bovis, C andersoni, C erinacei, C xiaoi, C fayeri, C scrofarum, C tyzzeri* and *Cryptosporidium cervine*, genotypes found in horses, rabbits, skunks, minks and chipmunks have also been isolated from humans.[5,6]

Although the global prevalence of *Cryptosporidium* is not well known worldwide, it is reported as the major factor causing severe diarrhea in children under 5 years of age in low-income countries.[7] Prevalence in patients with gastrointestinal symptoms shows variability based on geographic region. Accordingly, the prevalence of *Cryptosporidium* is between 1% to 4% in Europe and North America whereas it is 3% to 20% in Africa, Asia, Australia, South, and Central America.[8] In İzmir, the region in Turkey where samples were collected, the prevalence of *Cryptosporidium spp.* varies from 6.27% to 33.47%.[9,10] In epidemiological investigations, different methods have been used and according to the method used, the prevalence varies. The first choice of detection is generally routine microscopy methods such as acid-fast (AF) modified Ziehl–Neelsen staining and direct fluorescent antibody test (DFA); both have low sensitivity.[11] The other is ELISA (enzyme-linked immunosorbent assay), targeting the surface proteins of the parasite, which is accepted as the gold standard for antigen detection in stool samples.[12] The polymerase chain reaction (PCR) method is the third option, which has higher sensitivity and specificity, but at a higher cost.[12] A lot of genes such as the small subunit rRNA, *Cryptosporidium* oocyst wall protein (COWP), thrombospondin-related adhesive proteins, the 70-kDa heat shock protein (HSP70), and actin genes have been targeted using conventional PCR, nested-PCR and real-time PCR.[13] Indeed, at least two methods should be used in the diagnosis of cryptosporidiosis instead of a single method for more accurate results.[14,15] Timely and sensitive diagnosis of *Cryptosporidium spp.* in patients with malignant solid tumor receiving chemotherapy is of utmost importance since severe diarrhea may lead to exacerbation of cancer due to the interruption of the chemotherapy. This situation may be life-threatening for the patient. The present study aimed to investigate the prevalence of cryptosporidiosis in patients with malignant solid tumors receiving chemotherapy using microscopic, immunological and molecular methods to achieve accurate results for the prevalence in our study population.

## PATIENTS AND METHODS

Stool samples collected from adult patients with malignant solid tumors receiving chemotherapy were included in this study. All stool samples were collected from patients followed in oncology polyclinics. Each stool sample collected from patients was divided into three parts and then analyzed for the presence of *Cryptosporidium spp.* by microscopy, ELISA and real-time PCR. The study was approved by the Local Research Ethics Committee of Izmir Katip Celebi University Faculty of Medicine (Approval number: 27.09.2018 İKÇÜTF-106). Stool samples were concentrated by formalin-ethyl acetate concentration technique and stained by acid-fast (AF) modified Ziehl-Neelsen staining. Slides were examined at 1000x magnification under a light microscope to investigate the oocyst of *Cryptosporidium spp.*[16]

*Cryptosporidium spp.* antigen in stool samples was investigated by a commercial sandwich ELISA kit (RIDASCREEN, C 1201 r-biopharm, Germany) according to the manufacturer's procedure. Briefly, 100 µL of unconcentrated stool samples were diluted with 1 mL of sample dilution buffer, homogenized via a vortex mixer, and the stool homogenate was used in ELISA. The stool sample was considered positive if the absorbance value exceeded the negative control serum plus 0.15.

DNA was extracted from the stool samples using QIAamp DNA mini kit (Qiagen, USA) according to the manufacturer's protocol and all processes were performed manually. A real-time PCR targeting the 151-bp region of the *Cryptosporidium* oocyst wall protein (*COWP*) gene (GenBank no: AF248743.1) was applied using COWP-P702F (5′-CAAATTGATACCGTTTGTCCTTCTG-3′) and

COWP-P702R (5′-GGCATGTCGATTCTAATTCAGCT-3′) primers, and COWP-P702 hydrolysis probe (FAM-5′-TGCCATACATTGTTGTCCTGACAAATTGAAT-3′-BHQ) by a minor modification as described by Taniuchi et al.[17] Primers and probe were purchased from Metabion (Germany). Briefly, a 20-µL final volume PCR reaction contained a 5- µL template DNA, 0.5 µL of each primer (20 µM), 0.1 µL of the probe (20 µM), 4 µL of the TaqMan master mix (10×), and 9.9 µL of nuclease-free water. The PCR amplification reactions were performed by the following calculated protocol: 10 minute initial denaturation step at 95°C, followed by 45 cycles of 10 seconds at 95°C, 15 seconds at 55°C, and 15 seconds at 72°C. A positive control plasmid containing the *COWP* gene of *Cryptosporidium spp.* was prepared as described.[18,19] Positive controls contained 10-fold dilutions of positive control plasmid ranging from $6\times10^5$ to 6 copies of *COWP*/µL. One negative control, prepared by replacing template DNA with PCR grade water, was used in each run. Quantification analysis for each sample was performed by a 1.5 LightCycler Real Time instrument using LightCycler software, Version 4.0 (Roche, Germany). The stool samples which were positive by ELISA and negative by real-time PCR were also analyzed for the presence of inhibition by real-time PCR after 1:10 and 1:20 dilutions of the patient DNA samples.

*Cryptosporidium spp.* detection proportions among patients, which were categorized according to CD4+ T-cell counts (200-400 cells/mm$^3$, 400-800 cells/mm$^3$ and 800-1500 cells/mm$^3$) were computed and comparison of the proportions was performed by the chi-square test using the PASW Statistics version 18 software (*https://en.wikipedia.org/wiki/SPSS*). Statistically significant differences were $P$<.05.

## RESULTS

The 94 adult patients with malignant solid tumors receiving chemotherapy had a mean (SD) age of 64.1 years with 34 females and 60 males. All patients had diarrhea with a duration of more than 5 days. Patients with malignant solid tumor had colon cancer (n=28), lymphoma (n=13), pancreatic cancer (n=5), breast cancer (n=5), bladder cancer (n=5), stomach cancer (n=5), rectal cancer (n=4), lung cancer (n=4), basal cell carcinoma (n=3), multiple myeloma (n=3), prostate cancer (n=3), ovarian cancer (n=3), acute myeloblastic leukemia (n=3), laryngeal cancer (n=2), renal cell carcinoma (n=2), esophageal cancer (n=1), skin cancer (n=1), brain cancer (n=1), uterine leiomyosarcoma (n=1), malignant epithelial tumor (n=1) and hepatocellular carcinoma (n=1). Among these patients, CD4+ cell count was between 200-400 cells/mm$^3$ and 800-1500 cells/mm$^3$.

Oocysts of *Cryptosporidium spp.* were detected in two stool samples and the prevalence was 2.1% (2/94) by microscopy. *Cryptosporidium spp.* antigen positivity was detected in five stool samples and the prevalence reached 5.3% (5/94) (**Figure 1**). Only two stool samples were positive by both methods. Real-time PCR detected *Cryptosporidium spp.* in five stool samples and the molecular prevalence was 5.3% (5/94). Among real-time PCR positive samples, two inhibited PCR and one converted to positive after 1/10 dilution of the DNA sample, the other after 1/20 dilution of the DNA sample (**Figure 2**). Four *Cryptosporidium*-positive stool samples were positive by either microscopy-ELISA or ELISA-real-time PCR whereas the remaining four were positive by only ELISA or real-time PCR. Eventually, *Cryptosporidium spp.* was detected in eight stool samples according to results from three different methods. The prevalence of *Cryptosporidium spp.* was 8.5% (8/94) (**Table 1**). Using real-time PCR as a reference method, sensitivity and specificity for ELISA were 40% and 96.6% whereas positive predictive value and negative predictive value of ELISA were 40% and 96.6%, respectively. The accuracy was 93.6% for ELISA and the agreement between real-time PCR and ELISA was 94% (Cohen's kappa=0.36). A statistically significant difference in *Cryptosporidium spp.* detection proportion was not found among patients categorized according to CD4+ T-cell count.

## DISCUSSION

Due to the importance of cryptosporidiosis in cancer patients, the present study aimed to investigate the prevalence of *Cryptosporidium spp.* in patients with malignant solid tumors receiving chemotherapy. For this purpose, three different approaches analysing *Cryptosporidium*

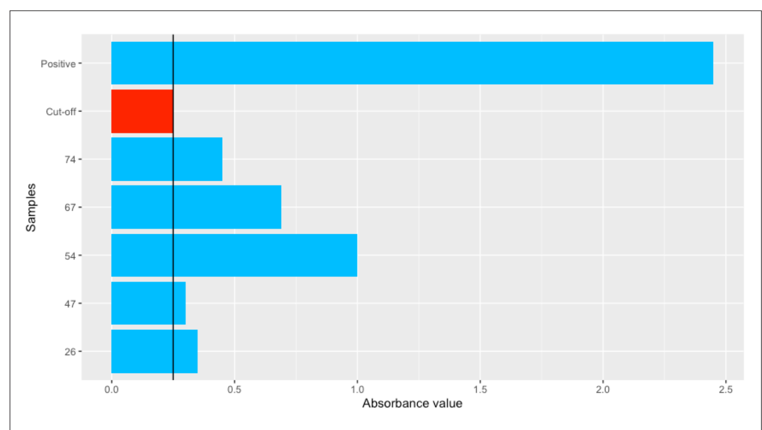


**Figure 1.** Absorbance values for ELISA-positive stool samples.

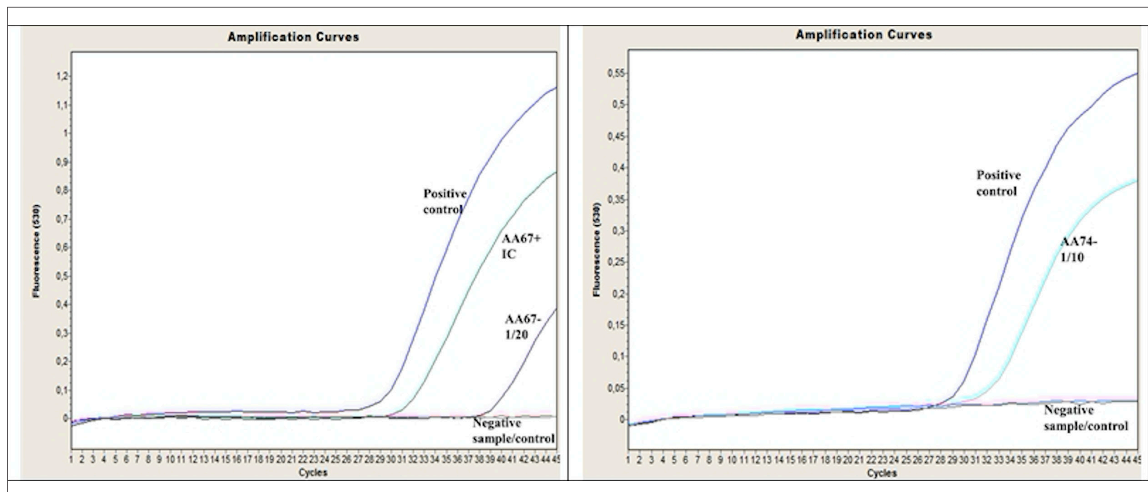


**Figure 2.** The second real-time PCR results for ELISA positive stool samples that were negative by first real-time PCR because of inhibition. In the right figure, the stool sample was positive in a 1/10 dilution whereas in the left figure, the stool sample was positive in 1/20 dilution. IC: inhibition control.

oocyst, antigen and DNA were used to reach more trustworthy and accurate results. When prevalence results obtained from each method were compared, the prevalence of *Cryptosporidium spp.* was 2.1%, 5.3%, and 5.3% by microscopy, ELISA, and real-time PCR, respectively. Moreover, the prevalence reached 8.5% by using three diagnostic methods. Although the prevalence (8.5%) detected in this study was low among patients with cancer and diarrhea, it shows the necessity of *Cryptosporidium spp.* control in this patient group when clinical outcomes caused by cryptosporidiosis are considered. In Turkey, the reported prevalence of cryptosporidiosis varies by study. One study reported that the prevalence of *Cryptosporidium spp.* was 17% in patients with neoplasia and diarrhea. In the same study, *Cryptosporidium spp.* was not detected in patients with neoplasia without diarrhea.[20] A similar prevalence value of 8.3% (similar to our results) was reported in a different study investigating *Cryptosporidium spp.* in patients with malignant solid tumors and diarrhea.[21] In countries other than Turkey several epidemiological studies have been carried out to understand the relationship of *Cryptosporidium spp.* and cancer, especially colorectal cancer in different countries. For example, Sulżyc-Bielicka et al (2007) reported that 10 out of 55 (18%) Polish patients with colorectal cancer had cryptosporidiosis according to their enzyme immunoassay results.[22] Two studies reported by Sulżyc-Bielicka et al (2012) and Sulżyc-Bielicka et al (2018), which used commercial immunoenzymatic test kits, showed simi-

**Table 1.** Clinical features of patients positive for *Cryptosporidium spp.*

| Sample no | Cancer type of *Cryptosporidium* positive patients | CD4+ cell count (cells/mm3) | Real-time PCR/CPT value | ELISA/absorbance value | Microscopy |
|---|---|---|---|---|---|
| 5 | Breast cancer | 800-1500 | Positive/36.92 | Negative/0.058 | Negative |
| 10 | Lymphoma | 400-800 | Positive/35.22 | Negative/0.045 | Negative |
| 14 | Colon cancer | 400-800 | Positive/35.86 | Negative/0.092 | Negative |
| 26 | Brain cancer | 400-800 | Negative | Positive/0.33 | Positive |
| 47 | Malignant epithelial tumor | 400-800 | Negative | Positive/0.24 | Negative |
| 54 | Lymphoma | 200-400 | Negative | Positive/0.99 | Positive |
| 67 | Colon cancer | 400-800 | Positive/38.29 | Positive/0.65 | Negative |
| 74 | Colon cancer | 400-800 | Positive/31.33 | Positive/0.37 | Negative |

CPT: crossing point threshold.Cut off value for ELISA was 0.21.

lar infection rates (12.6% and 13%, respectively) in patients with colorectal cancer.[23,24] Osman et al (2017) collected 218 biopsies from Lebanese patients and performed microscopic observation, immunofluorescence analysis and PCR, and reported that the prevalence of *Cryptosporidium spp.* was 21% in patients with colon adenocarcinoma whereas, the prevalence was 7% in patients without digestive cancers but with digestive symptoms.[25] Berenji et al (2007) used a modified Ziehl-Neelsen staining and ELISA to detect *C parvum* and the prevalence was 22% among children with lymphohematopoietic malignancies who underwent chemotherapy.[26] Sanad et al (2014) used a modified Ziehl-Neelsen staining method among 54 Saudi patients receiving chemotherapy for different malignant diseases and reported that prevalence of *Cryptosporidium spp.* was 61.5% in patients with breast cancer, 50% in patients with colon cancer, 100% in patients with colon cancer with metastasis, 50% in patients with liver cancer and 100% in patients with lymphoma.[27] Mousa et al (2014) performed microscopic examination after modified Ziehl-Neelsen staining and ELISA in 150 patients with chronic liver diseases and diarrhea. They reported that 32% of patients with hepatocellular carcinoma harbored *Cryptosporidium spp.*[28] Berahmat et al (2017) performed modified Ziehl-Neelsen (MZN) staining and PCR in 132 children with cancer undergoing chemotherapy. Using both techniques, they detected *Cryptosporidium spp.* in 5 patients; 2 had leukemia and 3 had other types of malignancy.[29] Zhang et al (2020) performed PCR to detect *Cryptosporidium spp.* among 195 patients with gastrointestinal cancers and reported that the prevalence of *Cryptosporidium spp.* was 17.24% in patients with colorectal cancer, 4% in patients with gastric cancer, 6.25% in patients with esophageal cancer, 14.29% in patients with liver cancer and 40% in patients with small intestine cancer.[30] On the other hand, some studies have reported an unexpectedly low prevalence of enteroparasites including *Cryptosporidium*, including none at all.[31,32] The findings of our study as well as previous research results highlight that this patient group is under risk of cryptosporidiosis and cases with diarrhea should be taken into consideration in terms of cryptosporidiosis.

In this study, the CD4+ T-cell counts were between 200-400 cells/mm$^3$, 400-800 cells/mm3 and 800-1500 cells/mm$^3$, and among these, the CD4+ T-cell count of a few patients (n=7) were 200-400 cells/mm$^3$, which reflects the risk of acquiring opportunistic infections including *Cryptosporidium spp.* Remarkably, one of these seven patients was positive. The prevalence of *Cryptosporidium spp.* can reach 14% among patients with 200-400 cells/mm$^3$. This result is also in line with a previously conducted study[27] and demonstrates the importance of the CD4+ T-cell in host resistance against *Cryptosporidium spp.* infection.

Another significant result in this study was the efficiency of using at least two methods in the diagnosis of cryptosporidiosis. The prevalence of *Cryptosporidium spp.* increased to 8.5% by this approach. The lowest prevalence was obtained by microscopy whereas the highest prevalence was detected by ELISA and real-time PCR. Weber et al reported that the sensitivity of the AF-modified Ziehl–Neelsen staining was 10 000 oocysts per gram of watery stool and thus concluded that the microscopic method can fail to detect cryptosporidiosis in many immunocompromised and immunocompetent individuals.[11] On the other hand, it was reported that ELISA was 10 times more sensitive than AF-modified Ziehl–Neelsen staining and accepted as the gold standard for antigen detection in stool samples.[12] Our study results also support ELISA as a more sensitive than AF-modified Ziehl–Neelsen staining in the routine diagnosis of cryptosporidiosis. ELISA should be used in addition to microscopy especially in immunocompromised patients with diarrhea.

In our study, the prevalence value obtained from real-time PCR targeting the *COWP* gene was the same as with ELISA. In a study conducted by Omoruyi et al the highest prevalence was obtained by ELISA compared to modified Ziehl-Neelsen and conventional PCR targeting the 18S rRNA gene.[12] In the same study, the prevalence as detected by ELISA and PCR was similar in HIV-negative patients with diarrhea whereas the positivity value of ELISA was prominently higher among HIV-positive patients with diarrhea.[12] Indeed, the PCR method is as sensitive ELISA but PCR inhibition caused by complex polysaccharides, bilirubin, or bile salts in stool samples can halt the diagnosis of the gastrointestinal parasites and this may cause false negative results.[33] In the present study, inhibition was detected in two stool samples that were positive by ELISA and after 1/10 or 1/20 dilutions of these DNA samples resolved the inhibition, these stool samples were also found positive by real-time PCR. This experience is actually very important for diagnostic studies and indicates that it may be beneficial to resolve the PCR inhibition. Depending on this suggestion, this situation can also be accepted as a limitation of our study since all real-time PCR negative samples were not studied at 1/10 or 1/20 dilutions.

In conclusion, these findings show the potential risk of cryptosporidiosis in cancer patients with diarrhea. Also, instead of a single method, it was demonstrated

that there is a need for at least two different methods including ELISA and PCR for the epidemiological survey or diagnosis of cryptosporidiosis to reach more accurate and trustworthy results. Moreover, it was concluded that the probability of inhibition should not be ignored during use of PCR methods.

**Author contributions**

*Conceived and designed the experiments: AAG, MK, HC Performed the experiments: MK, TÖÖ, HC, MD, SEA, ADD, MK, AEK, AA, BP, AG. Analysed the data: AAG, HC, CÜ, AYG, SK. Wrote the paper: HC, AAG, MD. Reviewed and edited the paper: AYG, CÜ, SK, ADD. All authors have read and approved the manuscript.*

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