RESEARCH ARTICLE

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Role of *Cryptosporidium* spp in Development of Colorectal Cancer

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Abstract

Background: Colorectal cancer is one of the most common malignancies in humans. About 20% of the cancer incidence was attributed to infectious agents highlighting the association between infectious agents and the development of cancers. It has been suspected that *Cryptosporidium* spp. infection may be correlated with colon adenocarcinoma. **Aim:** investigate the percentage of cryptosporidiosis among colorectal cancer patients. **Subjects:** 100 patients were recruited from Medical Research Institute, Alexandria University. **Methods:** Fresh stool specimens were collected, homogenized and examined using direct wet mount and by permanent staining of faecal smears using Modified ZN staining. Molecular detection by PCR amplification of *Cryptosporidium* COWP gene. **Results:** Significantly higher proportion of colorectal cancer patients (32.5%, 42.5%) tested positive by MZN and ELISA respectively compared to only 3.3% and 5% of positive MZN and ELISA among control group. Also, positive PCR was detected among higher proportion of colorectal cancer patients (47.5%) and only 5% of control group. Odds of colorectal cancer is 19 times among positive cases of *Cryptosporidium* by PCR than those without proven infection by PCR (OR 19.12; 95% CI 4.82-75.99). Comparison of the assessment of *Cryptosporidium* infection made by two techniques produces a kappa value of 0.770, and .759 respectively between NZN, ELISA and PCR as a gold standard, suggesting a good agreement between the two techniques and PCR. This value of kappa is significantly different from zero, K.770, p<0.001 for MZN and K.759, p<.001 for ELISA. Specificity of MZN (100%) is higher than that of ELISA (96.2%) and both reported higher specificity than sensitivity denoting that both tests are good positive to rule in the presence of infection at 40% prevalence. **Conclusion:** *Cryptosporidium* infection is significantly higher among cancer colon patients reinforcing that it might be considered as a likely risk factor for the development cancer colon.

Keywords: Cancer colon- *cryptosporidium*- ZN modified staining- ELISA and PCR

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Introduction

Cancer colon is a main cause of cancer related morbidity and mortality. It is ranked as the third most common cancer diagnosed cancer, and the third principal source of cancer-related death worldwide (Feng et al., 2021). In Egypt, cancer colon constitutes about 6.5% of all cancers and represents the sixth most common cancer diagnosed (Hassan et al., 2021). Chronic inflammatory conditions together with chronic long-standing infections and unhealthy diet and lifestyle are among the most common risk factors for its development (Taghipour et al., 2022).

It is a multifaceted diverse disease concerning its surgical site and genetic milieu. It has multiple etiological factors, one of the interesting causes is *Cryptosporidium* infection. Protozoans "*Cryptosporidium* genus" are intracellular intestinal parasites common in humans and animals. So far, more than 20 species of this genus can be distinguished, among them the *C. hominis* and *C. parvum* species which causes the human cryptosporidiosis (Sawant et al., 2020). It is one of the gastrointestinal parasites (GIPs), that represent a major health problem worldwide (Sepahvand et al., 2022).

Cryptosporidium is transmitted mainly through fecal-oral route, by ingesting living oocysts, fecally contaminating food or water. It can also be transmitted to humans through direct contact with infected persons (Gerace et al., 2019). In spite of the severe threat of *Cryptosporidium* infection to immunocompetent and immunocompromised individuals, there is still no ideal prevention and treatment method of cryptosporidiosis till

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now (Kalantari et al., 2019).

Cryptosporidiosis represents a major health problem as it is a frequent cause of diarrhea in both immunocompetent and immunodeficient individuals. The risk of developing a severe disease differs depending on the individual immune status. In immunocompetent humans, exposure usually results in a self-limited disease manifested by watery diarrhea with a duration of about 2 weeks (Sawant et al., 2020). In contrast, in immunocompromised patients as an acquired immune deficiency syndrome (AIDS), cancer chemotherapy, or organ transplantation in addition to pregnant women, preschool children, and infants are more susceptible to the spread of this organism and the parasite is of particular clinical significance as it may cause a severe persistent disease that may be life-threatening due to chronic diarrhea, water and electrolyte disorders, and absorption and nutrition disorders may develop (Sepahvand et al., 2022).

Cryptosporidium spp. infections have been suggested to be associated with several cancers, such as hematological malignancies, colorectal cancer, and liver cancer. It was postulated that *Cryptosporidium* spp. infections might be related to the development and progression of cancer colon via triggering colonic mucosal dysplasia (Taghipour et al., 2022).

Diagnosis of cryptosporidiosis has progressed from histological identification in intestinal biopsies to microscopic examination of fecal specimens for infective oocysts, enzyme immunoassay (EIA) for parasite antigens. Modified acid-fast staining technique is the commonly used stain for the detection of oocysts in fecal smears, however, the sensitivity and specificity appeared to be rather low, as identification depends on the experience and skills of the microscopist. The low sensitivity of diagnosis using staining techniques may limit early diagnosis, early treatment and possible prevention of the fulminate life threatening diarrhea seen in immunocompromised patients. Recently, molecular techniques have been used in diagnosing cryptosporidiosis in developed countries, owing to its sensitivity, specificity and rapid turnaround time (Campbell et al., 2022).

Materials and Methods

Subjects

Two hundred subjects were enrolled in the present study and was divided into; One hundred newly diagnosed cancer colon patients and 100 control subjects. Subjects were recruited from Clinical oncology department medical research institute; Alexandria university & was presented to Parasitology department. The study was conducted after the approval of the Medical Ethical Committee of the Medical Research Institute, Alexandria University in agreement with the Declaration of Helsinki and the International Conference on Harmonization Guidelines for Good Clinical Practice. Informed consent was taken from all subjects before the beginning of the research. Concealment of subjects' data was safeguarded for all the enrolled subjects.

Methods

Fresh stool specimens free from water and urine were collected from all subjects in clean, dry disposable plastic containers. Stool samples were homogenized by thorough mixing immediately after delivery to the laboratory. A portion of each sample was kept in a labeled clean test tube without preservative and stored at -20ºC for DNA extraction and PCR. Another portion was preserved in 10% formalin for microscopic examination.

Stool samples were prepared and examined using the following techniques:

• Direct wet mount: with saline and iodine (Arora and Brij, 2012).

• Permanent staining of fecal smears from concentrated fecal samples using Modified Ziehl- Neelsen staining. Figure 1.

• Molecular detection by PCR amplification of *Cryptosporidium* COWP gene from stool samples.

Stool samples were subjected to DNA extraction and PCR amplification of *Cryptosporidium* COWP gene. DNA extraction was done using the QIAamp® stool DNA isolation Mini Kit (Josefsen et al., 2015). Before extraction, each sample was subjected to eight cycles of freezing in liquid nitrogen for 1 min, followed by thawing at 98°C for 1 min to disrupt the oocyst wall (Elwin et al., 2012; Fayer et al., 2000; Gupta et al., 2008).

Polymerase chain reaction of the Cryptosporidium oocyst wall protein gene

The method relies on thermal cycling of repeated heating and cooling for DNA denaturation and enzymatic replication. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase are used for selective and repeated amplification (Bairami Kuzehkanan et al., 2011).

PCR master mix was obtained from Thermo Scientific (Thermo Scientific, UK- Maxima Hot Start Green PCR- 50 µL product no. K1081) containing: Taq DNA Polymerase, mg2+, dNTPs, water (nuclease-free) and the primers.

Two Primers for COWP gene were used, delimiting a 553 bp fragment. These were

Cry-1 5: 5 ′-GTA GAT AAT GGA AGA GAT TGT G-3′ and Cry-9: 5′-G GA C TG AAA TAC A GG C AT TAT CTT G-3′.

The amplification steps included initial denaturation step at 95ºC for 4 min, 30 cycles of denaturation at 94ºC for 50s, annealing at 54ºC for 30s, and extension at 72ºC for 50s, and final extension step at 72ºC for 10 min.

Positive and negative controls were included in each reaction to assess PCR amplification and nonspecific bands formation. The DNA concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Detection of PCR product using gel electrophoresis

Amplicons (amplified DNA) were visualized using electrophoresis apparatus (Mini-plate France) on 1.5% agarose gel (Promega V 312A), stained with ethidium bromide (Promega, G188A).

Agarose gel electrophoresis for the products of the nested PCR targeting *Cryptosporidium* oocyst wall protein

(COWP) gene of *Cryptosporidium* at 553 bp. Figure 2

Statistical methods

Statistical analysis was performed using IBM SPSS statistics program version 21. Quantitative data were described by mean and median as measures of central tendency & Standard deviation, minimum and maximum as measures of dispersion, while categorical variables were summarized by frequency and percent. Chi-square test was used to study significant association between two categorical variables. Fischer's exact significance was used if more than 20% of total expected cell counts <5 at .05 level of significance. Independent sample t test was performed to detect significant difference in the mean quantitative variables between two groups of patients after testing variable's distribution by Kolmogorov-Smirnov test (Kotz, 2006).

Kappa measure of agreement assessed concordance of isolates extracted by ELISA, MZN and PCR where sensitivity (SE = true positive/true positive + false negative) and specificity (SP = true negative/true negative + false positive), False positive rate, False negative rate, Positive predictive value, Negative Predictive value, were calculated. Unadjusted as well as adjusted odds ratios were calculated by multivariate logistic regression model to assess independent predictors to colorectal cancer. All statistical tests were two-sided and judged at 0.05 significance level (Field, 2013).

Results

One hundred subjects were included in the present study, divided into 40 newly diagnosed cancer colon (CC) patients and 60 apparently healthy volunteers of comparable socio-economic standard as the control group. The mean age of CC group was 47.08 years compared to 48.37 years in the control group. 66.7% of CC patients were males while the 66.7% of control group were males.

Significantly higher proportion of colorectal cancer patients (32.5%, 42.5%) were tested positive by MZN and ELISA respectively compared to only 3.3% and 5% of positive MZN and ELISA among control group (p<.001, <0.001). Also, positive PCR was detected among higher proportion of colorectal cancer patients (47.5%) and only 5% of control group (p<0.001) Table 1.

Moreover, the odds of colorectal cancer is 19 times among positive cases of *Cryptosporidium* by PCR than those without proven infection by PCR (OR 19.12; 95% CI 4.82-75.99) Table 2.

Finally, the Comparison of the assessment of *Cryptosporidium* infection made by two techniques produces a kappa value of 0.770, and .759 respectively between NZN as well as ELISA and PCR as a gold standard, which suggests a good strength of agreement between the two techniques and PCR. This value of kappa is significantly different from zero, K.770, p<0.001 for MZN and K.759, p<.001 for ELISA. Specificity of MZN (100%) is higher than that of ELISA (96.2%) and both reported higher specificity than sensitivity denoting that both tests are good positive to rule in the presence of infection at 40% prevalence (Table 3).

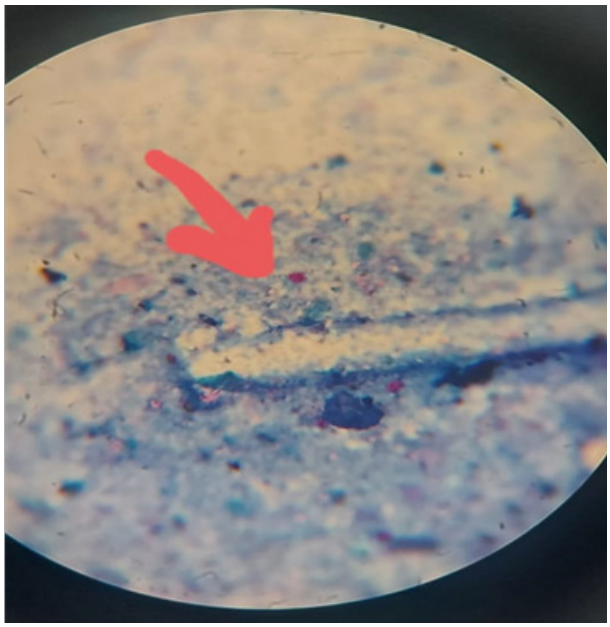

Figure 1. Cryptosporidium Oocyst by Modified Ziehl-Neelsen Staining (Arrow), Against a Blue-Green Background, the Oocysts Stand out in a Bright Red Stain (x1000)

Discussion

Gastrointestinal cancers are the most diagnosed cancers

Table 1. Demographic Characteristics of Colorectal Cancer Patients and Control Group

	Colorectal cancer pts (n=40)	Control (n=60)	Sig.
Age			
Mean± SD	47.08±12.93	48.37±11.70	0.605
20-40	11 (27.5%)	12 (20.0%)	
40-60	21 (52.5%)	39 (65.0%)	0.457
>60	8 (20.0%)	9 (15.0%)	
Sex			
Male	27 (67.5%)	40 (66.7%)	0.931
Female	13 (32.5%)	20 (33.3%)	
Residence			
Urban	21 (52.5%)	32 (54.2%)	0.865
Rural	19 (47.5%)	27 (45.8%)	
MZN			
-ve	27 (67.5%)	58 (96.7%)	<0.001*
+ve	13 (32.5%)	2 (3.3%)	
ELISA			
-ve	23 (57.5%)	57 (95.0%)	<0.001*
+ve	17 (42.5%)	3 (5.0%)	
PCR			
-ve	21 (52.5%)	57 (95.0%)	<0.001*
+ve	19 (47.5%)	3 (5.0%)	

MZN, modified Ziehl-Neelsen; ELISA, enzyme-linked immunosorbent assay; PCR, Polymerase Chain Reaction; *, Significant results≤.05

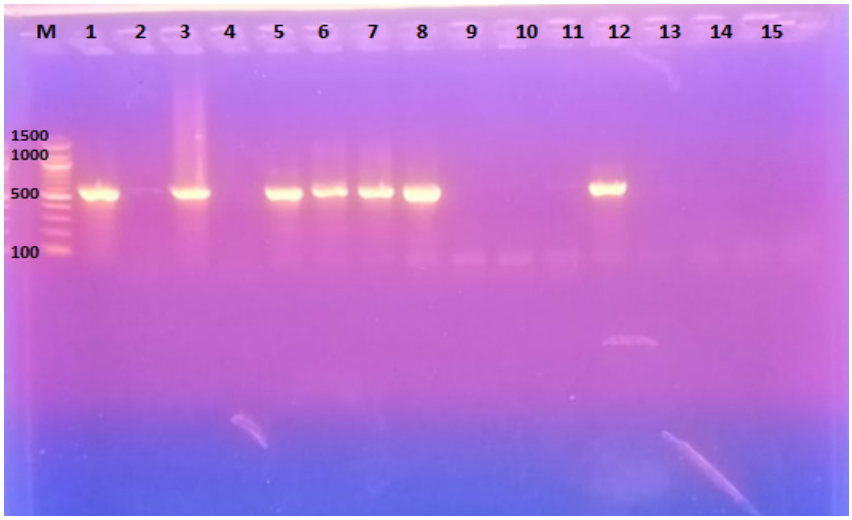

Figure 2. Agarose Gel Electrophoresis for the Products of the Nested PCR Targeting Cryptosporidium Oocyst Wall Protein (COWP) Gene of Cryptosporidium at 553 bp. Lane (M): DNA marker ladder. Lane 1 contained positive control, Lanes 3,5-8 and, 12: Positive for COWP gene while Lanes 2,4,9-11,13 and 14: are negative and lane 15 contained nuclease free water as negative control

Table 2. Multivariate Analysis to Assess the Independent Risk Factors of Colorectal Cancer Considering the Available Data

	Colorectal cancer pts (n=40)	Control (n=60)	Sig.
Age			
Mean± SD	47.08±12.93	48.37±11.70	0.605
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ELISA			
-ve	23 (57.5%)	57 (95.0%)	<0.001*
+ve	17 (42.5%)	3 (5.0%)	

X^2, 27.6; p<0.001*, R^2, 32.6%, model accuracy 76%; *, Significant results≤.05

and the leading cause of cancer deaths with over 30% for mortality in 2018. About 20% of the cancer incidence was attributed to infectious agents. Causal associations between infectious agents and the development of human cancers have already been highlighted. Overall, the total number of cancer attributable to infectious agents in 2002 was estimated at 1.9 million cases, standing for 17.8% of the global cancer burden. Moreover, it has been hypothesized that, by 2050, most human cancers could be due to infections (Sawant et al., 2020).

Colorectal cancer is one of the most common malignancy in humans considered the third highest neoplasm worldwide accounts about 9.7% of all tumors (Feng et al., 2021). It arises from an accumulation of genetic and epigenetic changes that transform healthy epithelial cells into cancer cells. *Cryptosporidium* spp. infections have been suggested to be associated with several cancers (Kalantari et al., 2019). It has been suspected that *Cryptosporidium* spp. infection may be correlated with colon adenocarcinoma (Sulżyc-Bielicka et al., 2018).

Our study illustrates the demographic as well as clinical characteristics of included colorectal cancer patients and control group. Both groups did not differ significantly in mean age (p.605). Nearly two third of both colorectal patients as well as control group were males (p.931). Also, the proportion of patients who live

Table 3. Difference in the Proportion of Positive Cases of Cryptosporidium, Agreement between MZN& ELISA, and PCR

	PCR results										PPV	NPV	Kappa measure of agreement
	(-ve) PCR			(+ve) PCR			Sp	Sn	FPR	FNR	(%)	(%)	
	-ve	+ve	N (-ve) PCR	-ve	+ve	N (+ve) PCR	(%)	(%)	(%)	(%)			
MZN	78	0	78	7	15	22	100	68.2	0	31.2	100	91.7	K.770, p<0.001*
ELISA	75	3	78	5	17	22	96.2	77.3	3.8	22.7	85	93.75	K.759, p<0.001*

Prevalence 40%; Sp, Specificity; Sn, Sensitivity; FPR, False positive rate; FNR, False negative rate; PPV, Positive predictive value; NPV, Negative Predictive value; K, Kappa Measure of agreement; *Significant results ≤0.05.

in rural areas did not differ significantly from controls (p.865). Significantly higher proportion of colorectal cancer patients (32.5%, 42.5%) were tested positive by MZN and ELISA respectively compared to only 3.3% and 5% of positive MZN and ELISA among control group (p<.001, <.001). Also, positive PCR was detected among higher proportion of colorectal cancer patients (47.5%) and only 5% of control group (p<0.001) (Table 2).

Osman et al., (2017) reported that the age of the patients was between 18 and 92 years (mean age: 50 ± 19). No significant differences related to age or sex were observed between groups. However, in patients with *Cryptosporidium* infection and colon neoplasia, the median age at time of diagnosis was lower compared to those patients with colon neoplasia without infection (X = 57,7 vs. X = 60.19). Patients with colon intraepithelial neoplasia/adenocarcinoma also had a 4 times higher risk of *Cryptosporidium* infection than patients without neoplasia but with other colon pathologies (OR: 4.05, CI: 1.39±11.79, P = 0.006). When the comparison was done using normal biopsies, the risk of infection increased 11-fold in the group of patients with colon adenocarcinoma (OR: 11.315, CI: 1.44±89.02, P = 0.003). *Cryptosporidium* infection was found only in colonic but not in gastric neoplasia samples, and this difference was significant (P = 0.014).

Zhang et al., (2020) found that 17.24% patients with colorectal cancer had C. parvum infections, which was significantly higher than control population. This infection rate was similar with the previous reports in colorectal cancer patients: 18%, 12.6% and 13% in Poland, and 21% in Lebanon.

In the present work, Kappa measures agreement between MZN, ELISA and PCR. Comparison of the assessment of *Cryptosporidium* infection made by two techniques produces a kappa value of 0.770, and .759 respectively between NZN as well as ELISA and PCR as a gold standard, which suggests a good strength of agreement between the two techniques and PCR. This value of kappa is significantly different from zero, K.770, p<.001 for MZN and K.759, p<.001 for ELISA. Specificity of MZN (100%) is higher than that of ELISA (96.2%) and both reported higher specificity than sensitivity denoting that both tests are good positive to rule in the presence of infection at 40% prevalence.

Morgan et al., (1998) reported that MZN staining has been found to be 97% specific with sensitivity of 66.6% as other studies which show MZN staining has been found to be 98.9-100% specificity with sensitivities ranging from 37-90% (Tuli et al., 2010). Kaushik et al., (2008) explained that with MZN staining, difficulties arise due to poor uptake of stain by oocysts sometimes, in discriminating between *Cryptosporidium* oocysts and other spherical objects of similar size (yeasts) staining dull red.

Several recent studies have shown an increased prevalence of *Cryptosporidium* in Poland, *Cryptosporidium* coproantigen was significantly more prevalent in patients with colorectal cancer than in a control group without malignant changes (Sulżyc-Bielicka et al., 2018). Similarly, Osman et al., (2017) showed that DNA and oocysts of *C. parvum* and *C. hominis* were more prevalent in digestive biopsies of patients with diagnosed colon neoplasia/adenocarcinoma compared to patients without digestive neoplasia.

In fact, the ability of *Cryptosporidium* spp. in the induction of digestive malignancies has been demonstrated in rodent models. Several studies showed that animal-derived C. parvum can induce the generation of colorectal, stomach and small intestine tumors using dexamethasone treated SCID (severe combined immunodeficiency) mice (Benamrouz et al., 2012; Certad et al., 2010; Gabriela Certad et al., 2010). Also, *C. parvum* of human origin can cause gastrointestinal and biliary adenocarcinoma in SCID mice. In contrast, another species *C. muris* did not induce the gastrointestinal cell transformation in the same experimental model, suggesting the unique role of *C. parvum* in the development of digestive cancers. Till now, more than 30 species of *Cryptosporidium* *Cryptosporidium* have been identified. C. parvum and C. hominis account for most of *Cryptosporidium* spp. infections in humans, while *C. meleagridis*, *C. felis*, *C. canis, C. muris, C. suis* and *C. andersoni* are occasionally observed in humans (Xiao, 2010). Previous molecular analyses indicated that *C. hominis* and *C. parvum* species were responsible for *Cryptosporidium* infections in colorectal cancer patient.

The pathological mechanism underlying *Cryptosporidium* spp. infections in the induction of digestive cancers remains unclear. However, available data suggested that *Cryptosporidium* spp., for its survival and transmission, can interfere with the cell signaling pathways and impact the gene expressions in host cells, thus, may secondarily promote the

generation of gastrointestinal cancers. It has been shown that C. parvum can inhibit the apoptosis of infected biliary epithelia through activating the NF-κB signaling pathway (Chen et al., 2001). A microarray analysis also revealed that C. parvum infection affected a large number of apoptosis gene expressions with upregulation of antiapoptotic genes and downregulation of proapoptotic genes at early stage (Liu et al., 2009). Resistance to apoptosis is one of the hallmarks of cancer development (Hanahan et al., 2011). Thus, *C. parvum* may induce the gastrointestinal cancers via preventing infected cells from cell death. Additionally, Benamrouz et al., (2014) found that *C. parvum* infections can alter the Wnt signaling pathway components and is able to modulate the cytoskeleton network in infected cells. They also showed that the changes of these biological processes were involved in the progression of ileo-caecal oncogenesis.

This parasite may result in a higher risk of developing colorectal malignancies and can generate invasive cancer in gastrointestinal and biliary epithelia of severe combined immunodeficiency (SCID) mice. Moreover, *Cryptosporidium* sp. is considered as one of the infectious agents that may induce intestinal dysplasia, including the high-grade category, which occurs particularly in the presence of immunosuppression states (Abdou et al., 2013).

Several scientists observed that when different groups of protozoa and helminths interact with their respective host, they caused DNA damage. In addition,

(Atwa et al., 2020) showed that *Cryptosporidium* sp. infection induced DNA damage in leucocytes of the chemically-immuno-suppressed mice; and the DNA damage was proportional to the severity of infection. Bhagat et al., (2017) reported that experimental infection with *Cryptosporidium* parvum in immunocompromised Swiss albino mice induced oxidative stress and elevated the lipid peroxidation, through enhancing the production of reactive oxygen species (ROS) and decreasing the antioxidant defense system. ROS produced by the infected host, as a defense strategy, not only caused the killing of the parasite but also induced oxidative DNA damage in uninfected cells.

The data show higher incidence rates in males than females. As there are relatively small differences in the data from routes to diagnosis to 5-year survival for males and females, it suggests the higher CRC death rate in males is primarily due to the higher incidence rate (White et al., 2018).

MZN staining has been found to be 97% specific with sensitivity of 66.6% which is in accordance with previous studies where MZN staining has been found to be 98.9-100% specificity with sensitivities ranging from 37-90% (Morgan et al., 1998; Tuli et al., 2010). These finding were supported by (Kaushik et al., 2008) who reported that with MZN staining, difficulties arise due to poor uptake of stain by oocysts sometimes, in discriminating between *Cryptosporidium* oocysts and other spherical objects of similar size (yeasts) staining dull red.

The PCR method proved to be more effective in determining *Cryptosporidium* in the stool samples. The primers used in our study work well and have high sensitivity and selectivity, thus preventing unwanted amplification products from being obtained. On the contrary (Amar et al., 2004) found less positivity by PCR as opposed to microscopy. The discrepancies between the present and other previous result may be due to differences in the primers used.

A study was done to determine the diagnostic efficacy of modified Ziehl-Neelsen (ZN), antigen detection ELISA, and a nested PCR assay for detection of *Cryptosporidium* in 58 adult AIDS cases with diarrhea from the ART clinic of Lok Nayak Hospital, New Delhi. *Cryptosporidium* was detected in 17 (29.4%), 39 (67.3%), and 45 (77.5%) cases by modified ZN staining, antigen ELISA, and nested PCR assay, respectively. Taking nested PCR as the gold standard, specificity of both modified ZN staining and *Cryptosporidium* antigen detection ELISA was 100% while the sensitivity of the tests was 37.8% and 86.6%, respectively. PCR was more sensitive than the other two diagnostic modalities but required a more hands-on time per sample and was more expensive than microscopy. (Uppal et al., 2014)

Molecular assays like PCR have the potential for accurate diagnosis in HIV seropositive subjects with diarrhea because of its high sensitivity. This will have considerable advantages in the treatment of AIDS patients, allowing early diagnosis before the onset of symptoms. PCR also has the added ability to directly differentiate between different *Cryptosporidium* genotypes, which assist in determining the source of cryptosporidial outbreaks. Sensitivity, specificity, ability to genotype, ease of use, and adaptability to batch testing make PCR a useful tool for future diagnosis and studies on the molecular epidemiology of *Cryptosporidium* infections. In spite of these advantages its widespread use is still hindered by its high cost and it remains till now confined to research purposes and epidemiological studies. However there exists a valid explanation for this assay to be routinely used for *C. parvum* diagnosis.

In conclusion, *Cryptosporidium* infection is significantly higher among cancer colon patients reinforcing that it might be considered as a likely risk factor for the development cancer colon. Although PCR is an expensive technique, it is fast, sensitive and specific for diagnosis of *Cryptosporidium* infections and can overcome the obstacles of traditional microscopic examination.

Author Contribution Statement

Naglaa Fathi Abd El-Latif: Selection of the research idea and research design, participation in the writing and reference gathering of the paper, participation in the performance of molecular laboratory tests, results, data interpretation, statistical analysis and plagiarism checking. Noha Said Kandil: Participation in the writing and reference gathering of the paper, participation in the performance of molecular laboratory tests, results and data interpretation, statistical analysis, plagiarism checking and paper revision and responsible for the international publishing process. Mohamed Shamsya: Participation in patient selection and participation in the writing and reference gathering of the paper. Yasmine Nagy Elwany: Participation in patient selection and collection of clinical data and participation in the writing and literature review and reference gathering of the paper. Heba Said Ibrahim: participation in the writing and reference gathering of the paper, participation in the performance of molecular laboratory tests, data interpretation and participation in the literature review.

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Declarations

Availability of data and materials

Data supporting the findings of this study are contained within the manuscript. The raw data are available by the corresponding author when requested.

Consent for publication

All authors agree for publication.

Ethics approval

• All patients were recruited from medical research institute teaching hospital. The study was approved by the

local ethics committee of the Medical Research Institute, in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) (reference number: IORG 0008812), for research involving humans, and informed consent was obtained from all included patients before commencement, explaining the investigational nature of this study.

• The study is approved by the Clinical Oncology Department Council, Medical Research Institute, Alexandria University.

• The study is not a part of an approved thesis or scientific body.

Conflict of interest

No conflict of interest to declare.

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