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ORIGINAL ARTICLE

Molecular prevalence of *Cryptosporidium* isolates among Egyptian children with cancer

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Abstract Immunocompromised individuals especially children with cancer are at risk for acquiring cryptosporidiosis, which can result in severe morbidity and mortality. This work was conducted to evaluate the prevalence of *Cryptosporidium* parasite and its genotypes in children with cancer. Stool specimens were collected from 145 children in the Oncology unit of Pediatric Department, Zagazig University Hospital, Sharqiyah province, Egypt. *Cryptosporidium* infection was evaluated using modified Ziehl–Neelsen (MZN) staining and nested polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis. The prevalence of *Cryptosporidium* infection in oncological children was 29.7% using microscopy and 25.5% using nested PCR. Genotypic characterization showed that 23 (62.2%) had *C. hominis*, 11 (29.7%) *C. parvum*, and 3 specimens (8.1%) were mixed infection of both genotypes. Cryptosporidiosis was significantly associated with diarrhea. However, no statistically significant difference was detected between the age, gender, residency, animal contact and malignancy type concerning to *Cryptosporidium* infection. This study concluded that *Cryptosporidium* is a prevailing opportunistic parasite among children with cancer. It should be considered in oncological patients especially those suffering from diarrhea which requires proper management to reduce its complications.

Keywords *Cryptosporidium* · Cancer · Children · Nested PCR · Modified ziehl–neelsen staining

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Introduction

Cryptosporidium, an apicomplexan parasite, is an important cause of child death globally. It results in a wide range of clinical features, from asymptomatic infection to severe illness (Kotloff 2017). The level of host immunity principally accounts for the different course of the disease, so the severity and duration of diarrhea and the other clinical symptoms of the infection differ in immunocompetent and immunocompromised individuals (Gerace et al. 2019). Other factors that affect the cryptosporidiosis manifestations include variability among parasite strains, coinfection with other intestinal pathogens, host nutritional status, the composition of gut microbiota, mucosal immune responses, and immune modulation (Certad et al. 2017).

Cryptosporidiosis is potentially life-threatening in patients with immune deficiencies, particularly Human immunodeficiency virus (HIV) patients or persons who have an organ or bone marrow transplant or are receiving chemotherapy. Diarrhea may become profuse and prolonged, resulting in wasting, while the water and electrolyte disturbances may be fatal (Sannella et al. 2019). In these immune-deficient patients, *Cryptosporidium* is usually spread to other organs including the pancreas, spleen, biliary tract, and respiratory tract (Sponseller et al. 2014). Children with malignancies are more susceptible to *Cryptosporidium* infection, with greater degrees of severity than immunocompetent cases (Ramadan et al. 2017). Also, Hijjawi et al. (2017) reported a high prevalence of cryptosporidiosis among pediatric oncology patients, it was about 2.8 times more than in non-oncological children. Moreover, cryptosporidiosis could be a possible risk factor for gastrointestinal tumors (Zhang et al. 2020).

Different approaches are available for the diagnosis of *Cryptosporidium*. Most commonly used diagnostic

techniques include detection of *Cryptosporidium* oocysts, antigens, or nucleic acid in stool samples. Other samples such as biopsy specimens, bile, duodenal fluid, bronchoalveolar lavage, and sputum can be used (Vanathy et al. 2017). The application of advanced molecular methods has led to better taxonomy and more understanding of *Cryptosporidium* phylogeny. Also, they are essential for assured diagnosis, a good understanding of molecular epidemiology, and for employing better control measures (Zahedi et al. 2016).

Studies are lacking in the molecular genotyping of *Cryptosporidium* species among oncological children in Egypt. In view of this, the study was conducted to understand the prevalence of *Cryptosporidium* species in the children with malignancy in Sharqiyah province for better management and control of cryptosporidiosis in these patients.

Patients and methods

Sample collection and ethical consideration

In this cross-sectional study, stool samples were collected from 145 children aging from 1 to 12 years, who were suffering from different types of malignancies and were hospitalized in the Oncology Unit of the Pediatric Department of Zagazig University Hospital, from February 2018 to August 2018. Patients taking antiparasitic drugs were excluded from the study. This study was ethically approved by the Institutional Review Board (IRB), Faculty of Medicine, Zagazig University. Before sample collection, informed consent, and a questionnaire including the demographic data and clinical symptoms were obtained from the parents or guardians of the involved children.

Microscopic examination

The Formalin ether concentration method was performed on portions of the stool specimens for microscopic analysis (Cheesbrough 2005). Modified Ziehl–Neelsen stained slides were examined for *Cryptosporidium* oocysts (Dubey et al. 1990). The remaining portions of the samples were stored at − 20 °C for molecular examination.

Molecular identification

Before extraction, the stool specimens were subjected to five freeze–thaw cycles of 95 °C for 5 min and − 80 °C for 5 min. DNA was extracted using QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) with modification in the form of prolongation of incubation to 95 °C for one hour (El-Badry et al. 2017). A Nested-PCR reaction was designed to target the COWP gene, using two primer pairs (Pedraza-Diaz et al. 2001). Thermocycling parameters were set following Spano et al. (1997). The amplification products were visualized by electrophoresis in 1.5% agarose gel containing ethidium bromide.

Cryptosporidium genotyping

RFLP analysis of the secondary PCR product was carried out by digesting with RsaI enzyme (Thermo Scientific Cat. No. ER1121), the obtained fragments were separated by electrophoresis on 2% agarose gel, stained with ethidium bromide, and visualized on an ultraviolet transilluminator.

Statistical analysis

All data were analyzed using SPSS 22.0 for windows (SPSS Inc., Chicago, IL, USA) & MedCalc 13 for windows (MedCalc Software bvba, Ostend, Belgium). Qualitative variables were expressed as frequency and percentage. The Chi-Square test was used to compare between groups for qualitative data. Kappa agreement was used for the instrumental agreement between MZN microscopy as a confirmatory test and PCR results of stool. All tests were two-sided. $P < 0.05$ was considered statistically significant (S), $P < 0.001$ was considered highly statistically significant (HS) and $P \geq 0.05$ was considered non-statistically significant (NS).

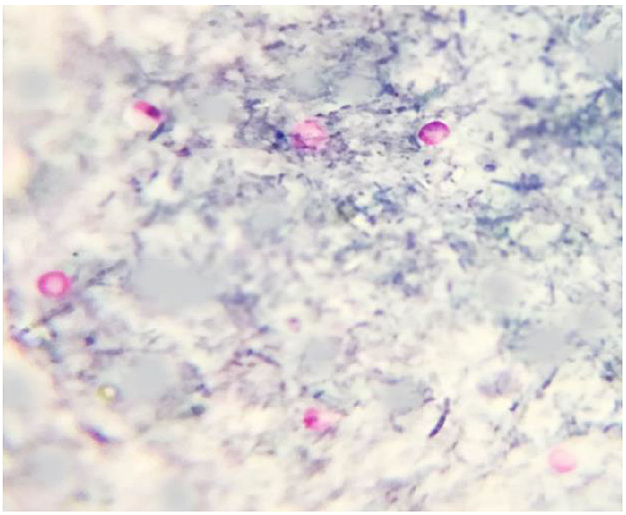

Fig. 1 Stool smear stained with MZN stain showing *Cryptosporidium* oocysts (X1000)

Results

Microscopic examination of MZN-stained stool specimens showed *Cryptosporidium* oocysts as rounded 4–6 µm pink to bright red findings against a bluish background (Fig. 1).

Out of 145 children with malignancies, MZN staining detected 43 *Cryptosporidium* positive cases and the nested PCR detected 37 positive ones (29.7% and 25.5% respectively). Upon evaluation of the two diagnostic techniques, there was a highly statistically significant association between both test results ($P < 0.001$) as well as an excellent agreement between them (kappa = 0.9) (Table 1).

In the current study, the MZN staining was considered the gold standard. Out of 43 positive cases by microscopy using MZN staining, PCR detected 37 cases (86.0%) of all positive cases by the MZN staining method. So, six cases could not be detected and considered false negative (14.0%). Regarding negative results, PCR excluded all negative cases (102) (100.0%) without false positives with sensitivity, specificity, predictive value positive (PVP), predictive value negative (PVN) and accuracy (86.0%, 100.0%, 100.0%, 94.4% and 95.8%) respectively as shown in Table 2.

PCR-RFLP analysis of PCR positive specimens (37) showed that existence of two genotypes: 23 (62.2%) had genotype I (*C. hominis*), 11 (29.7%) had genotype II (*C. parvum*), and 3 specimens (8.1%) were mixed infection of both genotypes (Fig. 2).

Among the patients' characteristics, a higher rate of *Cryptosporidium* infection was observed among children aged 5–8 years than the other age groups. Males were more affected than females and most of them were from rural residence but with no history of contact with animals. Concerning, the clinical manifestations, only the diarrhea was significantly associated (P value = 0.07) with *Cryptosporidium* infection (Table 3).

As seen in Table 4, there was no statistically significant difference regarding malignancy type between *Cryptosporidium* positive and negative cases (P value = 0.6).

Discussion

Cryptosporidium infection is associated with a diarrheal illness which leads to significant morbidity and mortality especially in children and immunocompromised individuals (Ojuromi and Ashafa 2018). It is one of the principal causes of diarrhea in children with cancer (Karim et al. 2020).

The detection rate of *Cryptosporidium* in the participating children with malignancies was 29.7% by the MZN staining method and 25.5% by nested PCR. A nearly similar percentage was previously obtained by Dyab et al. (2018) who reported that *Cryptosporidium* prevalence was 28% for oncological children using the MZN staining technique. However, a higher rate was recorded by El-Hady et al. (2017) who found that the rate of cryptosporidiosis in children underlying chemotherapy was 45% and attributed this association between cryptosporidiosis and cancer to the immunosuppressive effect of chemotherapy.

On the contrary, a zero-prevalence rate was stated by El-Badry et al. (2019) who attributed that to the good hygienic practices applied to oncological children who were receiving prophylactic anti-parasitic and antibacterial drugs. The variations in *Cryptosporidium* prevalence could be clarified by several factors such as type of cancer, immunosuppression effect of the chemotherapy, sociodemographic criteria, personal hygiene, sample size, nutritional status, seasonality and diagnostic technique used (Al-Warid et al. 2012).

It seemed that a higher detection rate of cryptosporidiosis was reported by the MZN staining method in comparison to the PCR procedure. So, this work showed that the nested PCR was less sensitive than the microscopic examination. Other reports noted a lower detection rate by PCR as opposed to microscopy such as Sadek (2014) and Kabir et al. (2020). This may be attributed to the failure of amplification of some fecal samples derived from reduced amounts of DNA, either due to its degradation or due to the low number of *Cryptosporidium* oocysts in positive fecal samples that yield inadequate DNA concentration to be

Table 1 Concordance (agreement) between microscopy and PCR in detection of *Cryptosporidium*

PCR	Microscopy (MZN)		Kappa agreement	*P* value
	Yes No. (43) %	No No. (102) %		
Cryptosporidium				
Yes (37)	37 (100.0%)	0.0 (0.00%)	0.9	0.001**
No (108)	6 (5.6%)	102 (94.4%)		

**Statistically highly significant difference ($P \leq 0.001$)

Table 2 The overall diagnostic ability of PCR in detection of *Cryptosporidium* comparing with microscopy (MZN)

Variable	Microscopy (MZN)				
	Sensitivity	Specificity	PVP	PVN	Accuracy
PCR	86.0%	100.0%	100.0%	94.4%	95.8%

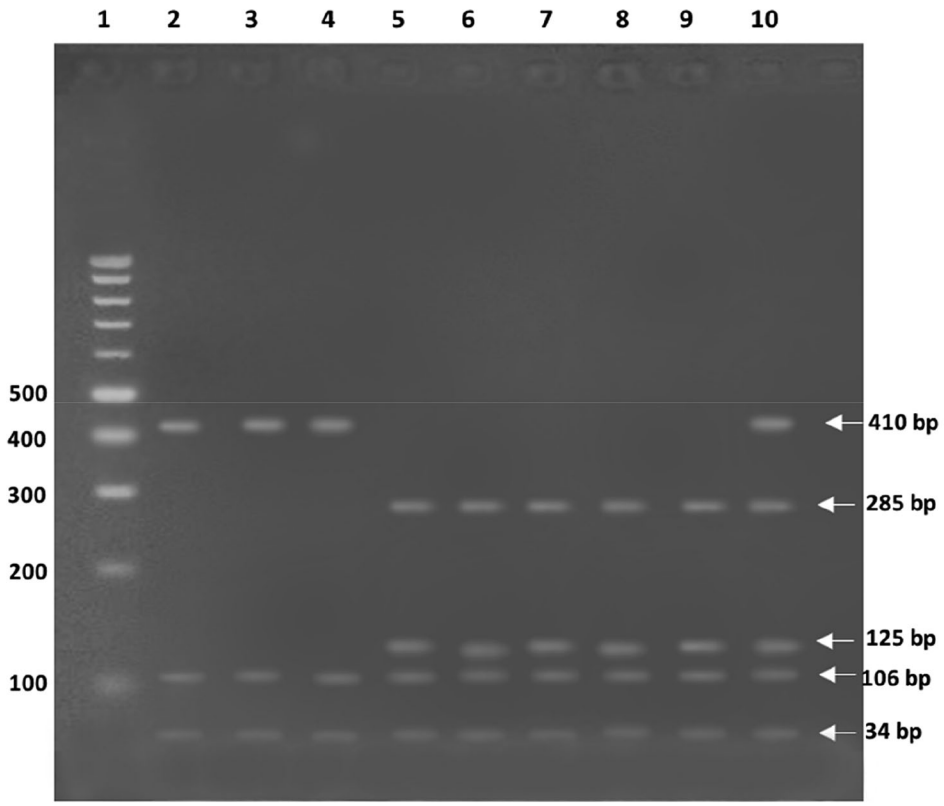

Fig. 2 Agarose gel electrophoresis of PCR-RFLP analysis for *Cryptosporidium* species. Lane 1: DNA ladder. Lanes 2, 3 and 4: *C. parvum* digestion products at 34, 106 and 410 bp. Lanes 5, 6, 7, 8 and 9: *C. hominis* digestion products at 34, 106, 125 and 285 bp. Lane 10: mixed infection digestion products at 34, 106, 125, 285 and 410 bp

detected by PCR (Wells et al. 2016). Al-Warid et al. (2019) found that the best detection limit of the PCR technique was more than 150 oocysts. Therefore, our false-negative PCR results could be due to the inadequate number of oocysts in stool samples and loss of DNA during the successive steps of washing and precipitation. Also, one of the main limitations for the sensitivity of PCR procedure is the presence of PCR inhibitors as polysaccharides, bile salts, hemoglobin, and lipids from food degradation products, mucus, and bacteria. These PCR inhibitors can act at different points: interfering with oocyst wall lysis, degradation or uptake of DNA, inactivation of Taq polymerase, and blocking the columns of DNA extraction (Abbaszadegan et al. 2007).

On the other hand, some studies suggest PCR detection of these protozoa is more sensitive than procedures such as microscopy and ELISA, for example, Geieth et al. (2019) reported that PCR allowed detection of infection among false-negative results by microscopic examination and ELISA. Also, Eassa et al. (2017) stated that PCR showed the highest detection rate for *Cryptosporidium* in stools collected from children with diarrhea. This discrepancy in reported PCR sensitivities may be related to numerous factors such as DNA extraction methods, gene region, levels of expertise, access to proper training, and the variety of sample types being examined e.g. fecal, water, or soil. To-date, a standardized molecular detection method that is rapid, affordable, and both specific and sensitive across a variety of sample forms still remains to be defined (Thompson and Ash 2019).

In the present study, nested PCR was used to increase the specificity of DNA amplification in which two sets of primers were involved in two successive reactions. Kuzehkanan et al. (2011), found that the nested PCR was better than conventional PCR as it detected one more case that was negative by both microscopy and conventional PCR. Moreover, Uppal et al. (2014) reported that nested

Table 3 Comparison between the positive and negative groups regarding patients' characteristics and their clinical presentations

Variables	Positive cases		Negative cases		$\chi 2$	*P* value
	No (43)	%	No (102)	%		
Age						
1–4 years	14	32.6	30	29.4	2.7	0.3
5–8 years	19	44.2	35	34.3		
9–12 years	10	23.2	37	36.3		
Gender						
Male	25	58.1	56	54.9	0.1	0.7
Female	18	41.9	46	45.1		
Residency						
Rural	35	81.4	77	75.5	0.6	0.4
Urban	8	18.6	25	24.5		
Contact with animals						
Yes	16	37.2	33	32.4	0.3	0.6
No	27	62.8	69	67.6		
Clinical presentations						
Diarrhea	25	58.1	35	34.3	7.1	**0.007***
Abdominal colic	20	46.5	50	49.1		
Systemic fever	5	11.6	23	22.5		
Vomiting	3	7	30	29.4		

*Statistically significant difference ($P < 0.05$)

Table 4 Malignancy type among the positive and negative groups

Malignancy type	Positive cases		Negative cases		$\chi 2$	*P* value
	No (43)	%	No (102)	%		
Hematological	38	88.4	80	78.4	1.9	0.1
Solid	5	11.6	22	21.6		

PCR was able to detect about 18% more positive samples than MZN staining and ELISA.

In the current work, PCR-RFLP analysis revealed that *C. hominis* was more dominant (62.2%) than *C. parvum* (29.7%). Mixed infections of both genotypes were recorded in 8.1% of PCR positive cases. This is in accordance with other studies such as Taha et al. (2018) and Naguib et al. (2018) who revealed the predominance of *C. hominis* in Egypt. Similarly, mixed genotypes were previously reported by Abd El Kader et al. (2012) and Gharieb et al. (2018). Conversely, Eida et al. (2009) and Eraky et al. (2014) reported that *C. parvum* was the predominant genotype.

Our study detected a high rate of cryptosporidiosis among children aged 5–8 years. These findings are in line with Shalaby and Shalaby (2015) and in contrast to Anejo-Okopi et al. (2016) who detected a high prevalence of *Cryptosporidium* infection among children aged 1.5–2 years.

Concerning gender, the infection rates did not vary with sex distribution. This result is consistent with Abdel Gawad et al. (2018). However, Adler et al. (2017) reported that males had a significantly higher risk of *Cryptosporidium* infection than females.

Regarding residency, children living in rural regions had a higher prevalence of this parasitic infection than those living in urban areas. Likewise, Steiner et al. (2018) and Liu et al. (2020) noticed high detection rates of cryptosporidiosis in rural areas.

In this study, there was no significant association between cryptosporidiosis and animal contact. Similarly, Quihui-Cota et al. (2017) and Boughattas et al. (2019) could not find a correlation with animal contact, suggesting the importance of other modes of transmission and perhaps

different species. On the contrary, El-Sherbini and Mohammad (2006) revealed a positive statistically significant association between farmers and their farm animals infected with *C. parvum* proving a zoonotic potential for the infection. Izadi et al. (2014) reported that cryptosporidiosis presents an occupational risk to individuals with cattle contact, consequently, there is strong evidence of zoonotic transmission from calves to humans.

Considering the clinical presentations, diarrhea was more common among *Cryptosporidium* positive than negative cases with a statistically significant difference. Similarly, Tombang et al. (2019) found *Cryptosporidium* infection was more common among children with diarrhea than non-diarrheic cases. Many studies described an association between watery diarrhea and the presence of *Cryptosporidium* infection as found by Elshahawy and AbouElenien (2019) and Gerace et al. (2019).

Pielok et al. (2019) reported that cryptosporidiosis must be taken under consideration in the differential diagnosis of persistent diarrhea as it could be responsible for gastrointestinal pathology with subsequent chronic diarrhea even in persons without immunological disorders. Conversely, Sadek (2014) noticed that most of *Cryptosporidium* positive individuals had formed stool. The shedding of *Cryptosporidium* oocysts by non-diarrheic asymptomatic carriers is considered a potential source of infection as these persons can spread the infection via bad personal hygiene or inappropriate sanitation to others (Reh et al. 2019).

In the present work, there was no statistically significant difference regarding malignancy type either hematological or solid between *Cryptosporidium* positive and negative cases (P value = 0.6). Similar observations were obtained by Berahmat et al. (2017) and Ramadan et al. (2017). Hijjawi et al. (2017) found that leukemia followed by lymphoma were the most prevalent types of malignancy in *Cryptosporidium* positive oncological children. Also, Dyab et al. (2018) noticed that the *Cryptosporidium* infection rate was higher in blood cancer than other types. However, Kalantari et al. (2020) detected that the occurrence of cryptosporidiosis was associated with colorectal tumors but not correlated to hematological malignancies.

In conclusion, *Cryptosporidium* is considered an important causative agent of diarrhea in children with cancer. So, there is a necessity of increasing the awareness of cryptosporidiosis among physicians and laboratory personnel to properly evaluate the burden of cryptosporidiosis in these patients.

MZN staining should be indicated as a routine laboratory technique in detection of suspected *Cryptosporidium* infected cases and confirmation with molecular techniques is recommended. Further studies about molecular characterization of *Cryptosporidium* are needed for determining the prevailing species and sub-species of *Cryptosporidium* for better understanding of transmission, invasion, and pathogenesis. More work is required to elucidate the interesting correlation between *Cryptosporidium* and cancer (especially gastrointestinal tumors) and its effect on apoptosis process and gene expression within the host cells. For the Pediatric oncological patients, a standard protocol of prophylactic anti-parasitic drugs, together with basic sanitary practices, as well as good nursing can be a simple preventive measure against parasitic infections.

Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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