Burden of intestinal parasites in a cohort of diarrheic Egyptian children: Predominance of *Cryptosporidium* using nested PCR assay

Original Article

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ABSTRACT

Background: Diarrheic children are more vulnerable to a variety of pathogens including gastrointestinal parasites. Cryptosporidiosis is a major etiology of chronic diarrhea in Egyptian children; it has both short- and long-term consequences for their growth and development.

Objective: The purpose of this study was to determine in a cohort of children with diarrhea the prevalence of *Cryptosporidium* spp., molecularly; and other intestinal parasites, coproscopically, and to assess the association between *Cryptosporidium* and patient characteristics. As well as to evaluate the usefulness of molecular assay in detection of cryptosporidiosis in diarrheic children.

Subjects and Methods: Fecal specimens were collected from 102 diarrheic Egyptian children, aged 12 years and under. All fecal specimens were examined coproscopically by wet mount prior to and after concentration, as well as permanent staining with modified acid-fast (MAF) for detection of intestinal parasites. Molecular assay using nested PCR (nPCR) was performed for detection of *Cryptosporidium* oocyst wall protein (*cowp*) gene. The association of patient demographics and clinical data with detection of *Cryptosporidium* spp. was determined.

Results: *Cryptosporidium* copro-DNA was detected in 12 (11.8%) cases, for 5 (4.9%) of which oocysts were detected by MAF coproscopy; 9 cases of *E. histolytica* complex and 7 cases of *G. intestinalis* were detected by coproscopy. Other than the measure of head circumference, none of the patient characteristics had a significant association (P=0.027) with the detection of *Cryptosporidium*.

Conclusion: There is a clear predominance of intestinal protozoa in diarrheic children, and *Cryptosporidium* spp. was the major enteric pathogen. Molecular assay should be included for the routine laboratory diagnosis of cryptosporidiosis.

Keywords: children; *Cryptosporidium*; diarrhea; Egypt; intestinal parasites; nested-PCR.

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INTRODUCTION

Diarrhea is the second major cause of death in children under the age of five years, with almost 1.7 billion cases worldwide[1]. Intestinal parasitic infections (IPIs) affect the digestion and reabsorption of nutrients, and result in increased loss of nutrients due to vomiting, blood loss, and/or diarrhea, all of which cause nutritional deficits. Similarly, as mentioned, children with nutritional deficiencies are more vulnerable to IPIs, because nutritional deficiencies can decrease children's immune responses[2]. Environmental enteropathic dysfunction is a wide spectrum intestinal disease characterized by nutritional malabsorption, intestinal inflammation, and disruption of barrier. Translocation of indigenous intestinal bacteria occurs through damaged epithelial lining caused by continuous exposure to a range of intestinal pathogens including *Cryptosporidium* and other intestinal protozoa[3].

Notably, IPIs are among the most frequent infections worldwide, particularly in developing countries like Egypt, and continue to be a public health concern[4]. In Egypt, various prevalences of IPIs were recorded, reaching as high as 60% for children 12 years and under[5-7]. *Cryptosporidium* is an obligatory intracellular protozoan parasite that infects the gastrointestinal tract (GIT) and is a leading cause of GIT morbidity and a top cause of mortality due to diarrhea for preschool children under the age of five in developing nations[8,9]. Cryptosporidiosis in children can have far-reaching repercussions beyond diarrhea. Both symptomatic and asymptomatic cryptosporidiosis are sometimes linked to malnutrition and stunted growth in children living in

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environments with limited resources[10,11]. Malnutrition can promote GIT infections in children, including *Cryptosporidium*. Cryptosporidiosis can prevent absorption of nutrients, leading to malnutrition, long-term infection, and vicious cycles of cryptosporidial reinfection[12]. In order to assess nutritional status, the nutritional monitoring system utilizes a variety of methodologies adapted to various settings, although the majority of them use replicated cross-sections of anthropometric surveys[13].

Diagnosis of cryptosporidiosis is routinely performed by conventional methods: coproscopy and copro-immunoassays, which lack sensitivity resulting in missing cases of cryptosporidiosis. Molecular assays have a high sensitivity and specificity with high diagnostic accuracy, in addition they can be used to identify *Cryptosporidium* spp.[14]. This study's main purpose was to detect *Cryptosporidium* spp. and associated intestinal parasites in a cohort of diarrheic Egyptian children. Also, to determine the association between occurrence of *Cryptosporidium* and the studied children's characteristics, as well as to evaluate the performance of various diagnostic methods.

SUBJECTS AND METHODS

This cross-sectional hospital-based prevalence study of cryptosporidiosis was conducted from May 2018 to February 2019 in Medical Parasitology Unit at Faculty of Medicine, Cairo University.

Study design: The study included collection of stool specimens from diarrheic Egyptian children attending the outpatient clinics of Abu El Rish Cairo University Hospital, Kasr Al-Ainy Faculty of Medicine, Cairo, Egypt. All fecal specimens were examined coproscopically, for detection of intestinal parasites, and molecularly, for detection of *Cryptosporidium* DNA.

Study populations: Fecal specimens were collected from 102 children of both sexes, aged 12 years and under, suffering from diarrhea, with or without GIT symptoms. Stool samples from children who had co-morbidities that could affect their immune status were excluded. The children's weight (to the nearest 0.1 kg), height (to the nearest 0.1 cm), and head circumference (to the nearest 0.1 cm) were measured and recorded for each child at the time of stool specimen collection.

Stool specimen and data collection: Single fecal specimens were collected from each child and placed in a dry, easy-to-use, leak-proof plastic container. For each specimen, a questionnaire covering demographics, growth, and clinical characteristics of patients were recorded.

Copro-parasitological microscopy: The collected specimens were investigated using coproscopy and molecular methods at the Lab of Molecular Medical Parasitology (LMMP) and the Diagnostic and Research Unit of Parasitic Diseases (DRUP), Faculty of Medicine, Cairo University. Fecal specimens were microscopically screened for intestinal parasites using a direct wet mount[15] before and after formol ether concentration procedure[15], as well as permanent staining with MAF stain[15] for detection of oocysts of intestinal coccidian protozoa, including *Cryptosporidium*.

Copro-nested PCR assay: Following initial thermal shock of fecal specimens[15] to disrupt *Cryptosporidium* oocysts walls, copro-DNA was extracted using the Favor Stool DNA Spin Columns Isolation Mini Kit (Favorgen Biotech Corporation, Taiwan) according to the directions of the kit. *Cryptosporidium* copro-DNA was amplified using nPCR) assay targeting *Cryptosporidium* oocyst wall protein (*cowp*) gene[16]. The nested reaction was carried out using two sets of primer pairs. The primer pairs, PCR reaction and reaction conditions were performed as described previously[16]. The first primer pair was used to create a DNA template for the nested reaction to boost the specificity of *Cryptosporidium* detection. The second set of primer pairs was utilized to anneal the previously produced amplicon (769 bp) for *Cryptosporidium*. After ethidium bromide staining, products from 2ry PCR (533 bp) were electrophoresed on a 1.5% agarose gel and examined with a UV trans-illuminator.

Statistical analysis: For statistical analysis, data was recorded using the statistical package software SPSS model 26 (Chicago, IL, USA). Data were tabulated, and descriptive statistics for quantitative variables were utilized using mean, range and the standard deviation. Descriptive statistics for qualitative variables were applied using frequency and percentage. Statistical significance using the Chi-square test, was considered when the *P* value was <0.05. Diagnostic performance, including sensitivity, specificity, positive and negative predictive values (PPV, and NPV), diagnostic accuracy, as well as Kappa agreement of the diagnostic tests, was calculated.

Ethical considerations: The research was approved by the ethical committee of the Faculty of Medicine at Al-Azhar University. All parents/guardians of the children were informed verbally about the research purpose, and the collection of specimens was completed after obtaining their consent. The treating physician was informed of the test results for the purpose of prescribing the appropriate treatment for patients.

RESULTS

***Cryptosporidium* spp. prevalence and diagnostic performance:** Using MAF stain of fecal smears, *Cryptosporidium* oocysts were detected in 6 patients (5.8%) whereas nPCR detected *Cryptosporidium* DNA

in 12 patients (11.8%) (Table 1, and Figure 1). Table (2) shows the diagnostic performance and accuracy of MAF coproscopy for diagnosis of cryptosporidiosis.

Demographic, growth, and clinical data of the study participants: The mean age of the 102 diarrheic children in our study was (3±2.98), with a male/female ratio of 1.2:1; 53.9% of them were males and 46.1% were females. Our study population was divided into three age groups, (1) infants (<1 year), (2) preschool children (>1-5 year), and (3) school children (>5-12 year). The majority were pre-school age-group (52%). Children from urban areas (66.7%) were more than those coming from rural areas (33.3%). Diarrhea was the presenting complaint for all study participants, and was often accompanied by other GIT symptoms, including vomiting for 38 (37.3%), fever for 31 (30.4%), abdominal pain for 68 (66.7%), and dehydration for 38 (37.3%) children.

The association of demographic and clinical data with detection of *Cryptosporidium* spp.: The association of study participant demographics, growth and clinical data was analyzed as predictors of the presence of *Cryptosporidium* in diarrheic patients. Quantitative data was reported as mean, standard deviation (SD), minimum, and maximum of weight (kilograms), height and head circumference (centimeters) (Table 3); and as number and percentage for qualitative data, including: age, sex, residence and clinical symptoms (Table 4). Except for head circumference of infected children, none of the data studied had significant association with detection of *Cryptosporidium* spp. in stool specimens.

Parasites detected among all study population and *Cryptosporidium* nPCR positive cases: Four parasites detected in the diarrheic fecal samples of 21 children (20.6%) were as follows: *Cryptosporidium* spp. (12 cases), *E. histolytica* complex (9 cases), *G. intestinalis* (7 cases) and *E. vermicularis* (one case) (Table 5). Of the 21 cases with parasitic infection, 20 were due to enteric protozoa, and one case was a helminth. Single parasitic infection with *Cryptosporidium* spp. was found in 9 cases, *E. histolytica* complex in 6 cases and *G. intestinalis* in 6 cases. Multiple parasitism was found in four cases: two co-infected with *Cryptosporidium* spp. and *E. histolytica* complex, one case co-infected with *Cryptosporidium* spp. and *E. vermicularis*, and one case co-infected with *E. histolytica* complex and *G. intestinalis*. (Table 5). Three cases of *Cryptosporidium* spp. were co-infected with *E. histolytica* complex cysts (2 cases) and *E. vermicularis* eggs (one case), with a statistically significant association (*P*=0.043) (Table 5).

Table 1. Conventional diagnostic MAF microscopy and nPCR among all study individuals.

		nPCR		Total No. (%)
		Positive No (%)	Negative No (%)	
MAF Microscopy	Positive	5 (4.90)	1 (0.98)	6 (5.88)
	Negative	7 (6.86)	89 (87.26)	96 (94.12)
Total		12 (11.76)	90 (88.24)	102 (100)

Table 2: Diagnostic performance and Kappa agreement of MAF coproscopy compared to PCR for diagnosis of *Cryptosporidium* among all study individuals.

	Cryptosporidium MAF coproscopy	
	Value	95% confidence interval
Sensitivity	41.7%	06.1-77.2
Specificity	98.9%	96.1-10.1
PPV	83.3%	45.3-12.1
NPV	92.7%	86.1-99.3
Diagnostic accuracy	92.2%	85.5-98.8
Kappa*	0.52	28.7-74.9

***Key for Kappa:** < 0=poor agreement, >0.00-0.20 = slight agreement, >0.20-0.40 = fair agreement, >0.40-0.60 = moderate agreement, 0.61-0.80 = substantial agreement and >0.80-1.0 = almost perfect agreement.

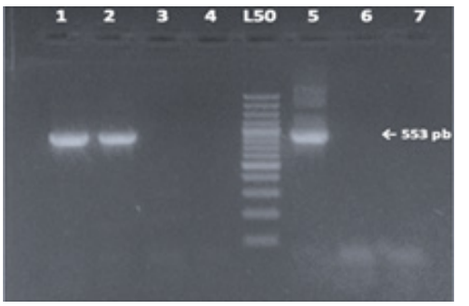

Fig. 1. The gel for nPCR products targeting *Cryptosporidium cowp* gene. **Lane L50** is molecular weight marker. **Lanes 1 and 2** are positive *Cryptosporidium* samples with a band at 553 bp. **Lanes 3, 4 and 7** are negative samples. **Lane 5** is positive control. **Lane 6** is negative control.

Table 3. Growth data (variables) for *Cryptosporidium* infected cases.

Variables		N	Min.	Max.	Mean	±SD	*P value*
Weight	PCR positive	12	3.00	22.00	10.08	4.32	0.772
	PCR negative	90	6.70	17.00	11.12	2.78	
Height	PCR positive	12	66.00	100.00	86.08	10.48	0.281
	PCR negative	90	50.00	140.00	81.52	19.77	
Head circumference	PCR positive	12	41.00	57.00	49.71	6.13	0.027*
	PCR negative	90	38.00	57.00	46.47	6.31	

N: number; **Min.:** Minimum; **Max.:** Maximum; **SD:** Standard deviation; ***P** value is statistically significant at <0.05

Table 4. Socio-demographic and clinical data of nPCR positive cases for *Cryptosporidium*.

Variables			Positive	Negative	Total	*P* value
			N. (%)	N. (%)	N. (%)	
Age group (Year)	**≤1**		1 (0.9)	24 (23.6)	25 (24.5)	**0.362**
	>1-5		8 (7.8)	45 (44.2)	53 (52.0)	
	>5-12		3 (2.9)	21 (20.6)	24 (23.5)	
Sex	**Male**		6 (5.9)	49 (48)	55 (53.9)	**0.772**
	Female		6 (5.9)	41 (40.2)	47 (46.1)	
Residence	**Urban**		8 (7.8)	60 (58.9)	68 (66.7)	**1.000**
	Rural		4 (3.9)	30 (29.4)	34 (33.3)	
Clinical manifestations	**Vomiting**	**Yes**	6 (5.9)	58 (56.8)	64(62.7)	**0.331**
		No	6 (2.9)	32 (34.4)	38 (37.3)	
	Abdominal pain	**Yes**	9 (8.8)	59 (57.9)	68 (66.7)	**0.81**
		No	3 (2.9)	31 (30.4)	34 (33.3)	
	Fever	**Yes**	4 (3.9)	29 (26.5)	31 (30.4)	**076**
		No	8 (7.8)	63 (61.8)	71 (69.6)	
	Dehydration	**Yes**	7 (6.9)	31 (30.4)	38 (37.3)	**0.267**
		No	5 (4.9)	59 (57.8)	64 (62.7)	
Total			**12 (11.8)**	**90 (88.2)**	**102 (100)**	

P value is statistically significant at <0.05

Table 5. Parasites detected in study population.

	Coproscopy			*P* value
	nPCR positive (N)	nPCR negative (N)	Total [N. (%)	
Parasites				
G. intestinalis cysts	0	6	6 (5.8)	**0.043***
E. histolytica complex cysts	2	6	8 (7.8)	
E. histolytica complex + *G. intestinalis* cysts	0	1	1 (0.9)	
E. vermicularis eggs	1	0	1 (0.9)	
Total	3	13	16 (15.7)	
No parasites	9	77	86 (84.3)	
Total	**12 (11.8)**	**90 (88.2)**	**102 (100)**	

P* value is statistically significant at <0.05; **N: Number; *E. histolytica/dispar/moshkovskii* complex.

DISCUSSION

The vicious cycle of diarrhea and malnutrition has been identified as a major contributor to significance of child morbidity and mortality in impoverished areas of the world. Infection with *Cryptosporidium* spp. leads to chronic diarrhea and malnutrition, especially in young children. The relationship between subclinical cryptosporidiosis and malnutrition is now well established, although not completely understood[10,11].

In our study, 12 diarrheic children were positive for cryptosporidiosis (11.8%). This result coincides with the 10.4% prevalence found in Ethiopia[17] and is higher than previously recorded findings from Egypt by Elmatrawy *et al*.[18] and Ibrahim *et al*.[19], who found *Cryptosporidium* in 6% and 8.8% of Egyptian children respectively. In Egypt, higher *Cryptosporidium* prevalence using PCR were found in about one fifth of diarrheic Egyptian children[16,20]. The difference in prevalence of *Cryptosporidium* between previous studies and the current study could be attributed to variances in population's demographic characteristics, socioeconomic levels and growth curves, as well as differences in sample sizes, diagnostic methodologies and seasonality and duration of study period[10,11,16,20].

Cryptosporidium has no standard diagnostic method[21]. Because of its higher sensitivity and specificity than the acid fast stain coproscopy, the nPCR assay was chosen as the reference standard method in this study[16,19]. The MAF stain identified *Cryptosporidium* oocysts in only 5 (4.9%) of the cases in our study, with a low sensitivity (41.7%) and excellent specificity (98.9%). The MAF stain may generate false results due to low parasite load, small size of *Cryptosporidium* oocysts, and difficulty in the microscopic detection of their interior and/or exterior features, increasing the chance of misinterpretation[16].

In the current study, we found that the head circumference showed statistically significant association with *Cryptosporidium* infection (*P*=0.027). While preschool children were apparently more frequently infected than the other two age groups, there was no statistically significant association (*P*= 0.362). The mean age of *Cryptosporidium* positive cases was 4 years, ranging from 1 to 8 years. According to a study

by Derouin *et al*.[22], cryptosporidiosis has a particularly negative impact on children in their first four years of life. The prevalence of *Cryptosporidium* was the same in males and females in our study (5.9%), and there was no correlation between its prevalence and sex. Similarly, other studies[16,23] found no significant link between *Cryptosporidium* infection and sex. In the United States, Painter *et al*.[24] showed that males had a higher incidence of cryptosporidiosis than females for individuals under the age of 15, with no statistically significant difference. Male predominance was also reported in Egypt, and France[25,26]. In the present study, the infection rate of *Cryptosporidium* in urban areas was higher (7.8%) than in rural areas (3.9%). In contrast, in England and Wales, cryptosporidiosis was more frequent in agricultural rural areas, where there was more exposure to manure, as well as regions with inadequately treated water supplies[27]. According to Al-Shamiri *et al*.[28], 43.6% of cases in Yemen came from rural areas, whereas 25.1% came from urban areas. This possibly could be related to hygiene practices in rural areas.

In the current study, because diarrhea was a constant symptom, no statistical evaluation could be generated. None of the investigated clinical variables including vomiting, fever, abdominal pain and dehydration, were linked to *Cryptosporidium* infection. Asymptomatic cryptosporidiosis might cause slowing of weight gain and stunting growth in children[10,11]. Unfortunately, physicians rarely order stool examination for enteric infections in the case of malnourished infants who do not present with diarrhea and consequently are not treated.

In the current study, the frequency of IPIs in diarrheic Egyptian children was 20.2% (21 cases); four cases had multiple parasitism and three of them were co-infected with *Cryptosporidium* spp., with a statistically significant association (P=0.043). Protozoal parasites were the predominant intestinal parasites (19.3%), with only one case of helminthic infection (0.9%). *Cryptosporidium* was the major enteric protozoan infection (12 cases), followed by *E. histolytica* complex (9 cases), and *G. intestinalis* (7 cases). *Blastocystis* spp. was not detected in stools of any of the children studied.

Our results support prior research that also recorded these three intestinal protozoa: *Cryptosporidium* spp., *G. intestinalis*, and *E. histolytica* complex as the most common causes of diarrhea in children in Egypt, and other developing countries[29,30]. Additionally, two studies[31,32] observed that infections with *H. nana* and *E. vermicularis* were the most reported enteric helminthic infections. This finding of high prevalence of intestinal parasites is consistent with recent studies conducted in Egypt, reporting a 30.7% rate of IPIs among school-children in Damietta[6], 31% in Aswan[7]; in contrast, a much higher prevalence (60%) was reported in a study conducted in Greater Cairo in children under the age of 12[5]. The difference in prevalence of intestinal parasitism may be explained by variances in sample size, geographical areas and the diagnostic methods used.

In conclusion, based on our findings, there was a predominance of intestinal protozoa among the Egyptian diarrheic children, and *Cryptosporidium* spp. was the major enteric pathogen. Low sensitivity limits coproscopic detection of *Cryptosporidium* and can lead to misdiagnosis. Consequently, searching for *Cryptosporidium* spp. in routine work up using PCR assay is recommended.

Author contribution: Abdel-Maogod AA and El-Badry AA proposed the study topic and planned the study design. Abdel-Maogod AA contributed with Ibrahim A in the practical work, writing of original draft, and data evaluation. Bayoumy AS shared with Hassan KAM, El-Faramawy MS and El-Badry AA the conceptualization, validation, and supervision. All authors reviewed the final version.

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