Original Article

Genotyping of *Cryptosporidium* species in children suffering from diarrhea in Sharkyia Governorate, Egypt

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Abstract
Introduction: The protozoan parasite *Cryptosporidium* is one of the principal reasons for childhood diarrhea around the world. This work aimed to differentiate *Cryptosporidium* species among children suffering from diarrhea in Sharkyia Governorate, Egypt.
Methodology: A total of 97 fecal specimens were taken from children suffering from diarrhea, attending Pediatric Clinics of Zagazig University and Al-Ahrar Hospitals. Full history was taken. Stool samples were examined microscopically using modified Ziehl–Neelsen stain for detection of *Cryptosporidium* oocysts. To identify *Cryptosporidium* genotypes, positive samples were then subjected to nested Polymerase chain reaction-restriction fragment length polymorphism targeting *Cryptosporidium* oocyst wall protein gene.
Results: The overall detection rate was 27.8% (27/97) using modified Ziehl–Neelsen stain staining method. Using nested polymerase chain reaction, the gene was amplified in 85.2% (23/27). Restriction fragment length polymorphism analysis revealed that 65.2% (15/23) were *Cryptosporidium hominis*, 30.4% (7/23) were *Cryptosporidium parvum,* and one sample was not typed (4.4%). The significant risk factors associated with *Cryptosporidium* infection in children were animal contact and residence in rural areas.
Conclusions: *Cryptosporidium* is a common enteric parasite affecting children in Sharkyia Governorate, Egypt, with the predominance of *C. hominis* genotype in children.

Key words: Children; COWP gene; *Cryptosporidium*; genotyping; modified Ziehl–Neelsen stain; PCR-RFLP.

Introduction

Cryptosporidium is a protozoan parasite that has been recognized as a predominant and virulent agent of childhood diarrhea [1]. Early childhood cryptosporidiosis is accompanied by malnutrition, delayed growth, impaired immune response and cognitive deficits [2].

Cryptosporidium infection may be acquired through contaminated water, contaminated food, direct contact with infected persons or infected animals [3]. It has been recognized as a main organism in contaminated drinking water as it is resistant to conventional drinking water treatment procedures [4].

Cryptosporidium has a wide variety of hosts, as well as human, domestic and wild animals [5]. Recently, more than 34 species and 40 genotypes of *Cryptosporidium* have been recognized [6]. In humans, more than 20 species have been identified but the most causative species isolated from human are *C. hominis* and *C. parvum*. However, other species such as *C. meleagridis*, *C. canis*, *C. felis*, *C. muris*, *C. viatorum*, and *C. suis* can also infect human [7].

Better recognition of the epidemiology, sources and transmission of cryptosporidiosis is fundamental to improve the control programs. More data on case exposures, genotypes and subtypes identified by molecular studies would definitely afford more understanding of the pathogenicity and epidemiology of the parasite with better preventive measures [8].

Despite the high prevalence of *Cryptosporidium* particularly in developing countries, molecular characterization of its species is deficient and only few genetic and epidemiological studies have been done [9].

Therefore, this work aimed to detect the *Cryptosporidium* genotypes that infect children suffering from diarrhea in Sharkyia Governorate, Egypt.

Methodology

Ethical statement

This work was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Ethical approval to conduct this study was obtained from the Institutional Review

Board (IRB), Faculty of Medicine, Zagazig University, Egypt (approval number 4001/24-9-2017). Written informed consent was obtained from the parents of all the participated children.

Study design and sampling

This cross-sectional study was conducted from March-August 2018. Fecal specimens were taken from 97 children of ages 1 to 15 years, suffering from diarrhea, recruited from Pediatric Inpatient Sections and Outpatient Clinics of Zagazig University and Al-Ahrar Hospitals. Full history was taken from the parents of the children including age, sex, residence, animal contact, drinking water source and clinical manifestations.

Each stool specimen was divided into two portions. The first portion was preserved in formalin 10% for microscopic examination and staining. The second portion was stored at -20 °C for a maximum period of 5 months and was subjected to molecular examination.
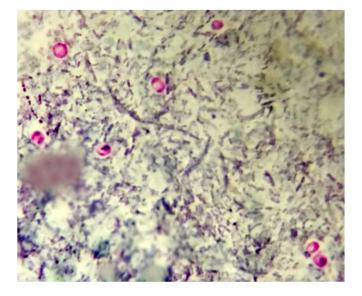
Microscopic examination of stool specimens

Each fecal sample was examined by Direct smear and Lugol's iodine stained smear [10] for detection of any parasitic stages using low and high-power objective lenses (10× and 40×).

Concentration and staining methods

Stool specimens were concentrated by formalin ether concentration technique [11], and smears were stained by modified Ziehl–Neelsen stain (MZN) stain [12]. The stained smears were examined thoroughly under the oil immersion lens.

Figure 1. *Cryptosporidium oocysts* (MZN stain, ×1,000).

Molecular examination

Molecular examination was performed at the Veterinary Genetics and Genetic Engineering Lab, Faculty of Veterinary Medicine, Zagazig University. Only the positive specimens by microscopic examination, were subjected to molecular examination.

Deoxyribonucleic acid (DNA) Extraction

Before extraction, each sample was exposed to eight freeze-thaw cycles (deep freezing in liquid nitrogen for 5 minutes, followed by thawing at 98 °C for 5 minutes). QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) was used to extract the DNA from the stool specimens, according to the manufacturers' protocol with modification in the form of increasing the lysis temperature to 95 °C and prolongation of the incubation time of the InhibitEX tablet step to 5 minutes [13].

Nested PCR

The nested PCR was performed targeting *Cryptosporidium* oocyst wall protein (COWP) gene using two consecutive PCR reactions. The primers were used for these reactions described by Pedraza-Díaz *et al.* [14]. The amplification mixture and conditions for both PCR reactions were accomplished according to Spano *et al.* [15]. The PCR products were electrophoresed on 2% agarose gel and visualized by ethidium bromide staining.

Restriction fragment length polymorphism (RFLP) analysis

The nested PCR products were digested with RsaI restriction enzyme (Thermo Scientific Cat. No. ER1121) following the manufacturer's protocol [15]. The digested PCR products were electrophoresed on 2% agarose gel containing ethidium bromide and visualized by an ultra violet transilluminator.

Statistical analysis

Analysis of data was done using SPSS software version 23. Chi-square test (χ^2) was applied to estimate the differences in proportions between variables. Fischer's exact test (FET) was used to compare categorical outcomes if one of the cells is less than 5. The *p* value less than 0.05 was considered as significant level.

Results

Screening of 97 children's fecal smears by MZN stain revealed 27 positive *Cryptosporidium* samples (27.8%). *Cryptosporidium* oocysts appeared as round or

ovoid 4–6 μm bright pink structures against bluish background (Figure 1). The positive samples by MZN stain were subjected to nested PCR targeting COWP gene. The gene was successfully amplified in 23/27 (85.2%). Nested PCR-RFLP analysis revealed the presence of 2 genotypes: 15 (65.2%) had genotype I (*C. hominis*), 7 (30.4%) had genotype II (*C. parvum*). One specimen (4.4%) could not be digested by *Rsa* I restriction enzyme (Figure 2).

Among the studied variables, only residence and animal contact were associated with cryptosporidiosis with a significant level ($p < 0.05$) (Tables 1 and 2). The percentage of infection was significantly higher in children inhabiting rural areas (85.2%). Also, *Cryptosporidium* detection was significantly higher among children with a history of animal contact (66.7%) (Table 1). None of the other studied variables, including age, sex distribution, drinking water source, stool consistency, and clinical manifestations, showed any significant association with the detection of *Cryptosporidium* infection ($p > 0.05$) (Tables 1 and 2).

Regarding *Cryptosporidium* genotypes, none of the variables showed significant difference between the *C. parvum* and *C. hominis* positive cases ($p > 0.05$) (Table 3). Other parasites were also detected during the microscopic examination of the stool specimens (Table

Figure 2. Agarose gel electrophoresis for PCR-RFLP. Lane M is the DNA marker. Lane 1,2,3 and 4: *C. hominis* digestion products at 34, 106, 125 and 285 bp. Lane 5 and 6: *C. parvum* digestion products at 34, 106 and 410 bp (34 band is indistinct and hard to be seen). Lane 7: undigested product of COWP product at 553 bp.
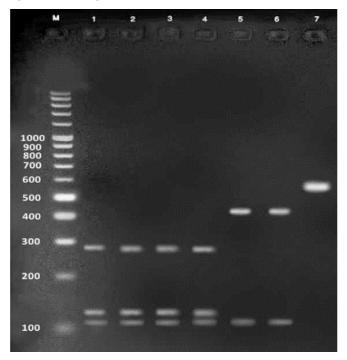

Table 1. Comparison of the demographic data between positive and negative cases according to microscopic examination results.

Variables		Positive cases		negative cases		χ^2	*p* value	Odds ratio (95% CI)
		(No = 27)	%	(No = 70)	%			
Age	1-5 years	13	48.1	24	34.3			
	6-10 years	9	33.3	30	42.8	1.5	0.4	NA
	11-15 years	5	18.6	16	22.9			
Sex	Male	15	55.6	37	52.9	0.5	0.8	0.89 (0.36-2.4)
	Female	12	44.4	33	47.1			
Residence	Urban	4	14.8	32	45.7	7.9	0.004*	4.8 (1.5-15.4)
	Rural	23	85.2	38	54.3			
Animal contact	Yes	18	66.7	27	38.6	6.1	0.01*	0.3 (0.1-0.7)
	No	9	33.3	43	61.4			
Water source	Tap	23	85.2	57	81.4			
	Underground	3	11.1	5	7.2	1.6	0.4	NA
	Filtered	1	3.7	8	11.4			

NA: not applicable; * $p < 0.05$ is significant.

Table 2. Comparison of stool consistency and clinical manifestations between positive and negative cases.

Variables		Positive cases		Negative cases		χ^2	*p* value
		(No = 27)	%	(No = 70)	%		
Stool consistency	Watery	9	33.3	16	22.9		
	Loose	12	44.5	25	35.7	3.2	0.2
	soft	6	22.2	29	41.4		
Clinical manifestation	Diarrhea only	9	33.3	16	22.9		
	Abdominal pain	8	29.6	23	32.8		
	Abdominal pain and fever	5	18.5	14	20	1.2	0.8
	Abdominal pain and vomiting	4	14.8	13	18.6		
	Abdominal pain and flatulence	1	3.8	4	5.7		

4). Four of the children infected with *Giardia lamblia* were also positive for *Cryptosporidium*.

Discussion

Cryptosporidium is increasingly recognized as a chief cause of diarrheal illness. Its largest burden occurs mainly in children inhabiting low-income regions [16].

In the current study, stool samples from 97 children were collected between March and August 2018. The authors preferred collecting specimens in the spring and summer months as many studies reported higher rates of *Cryptosporidium* infection in these seasons. Like, in Egypt, Abd El Kader *et al.* [17] stated that the maximum *Cryptosporidium* prevalence was in August than in February to March. The summer seasonal peak could be due to increased use of swimming pools, rivers, and lakes [18]. In late winter and early spring, de Lucio *et al.* [19] observed a high prevalence peak of cryptosporidiosis. However, no significant seasonal variation was reported by Tangtrongsup *et al.* [20].

The infection rate of cryptosporidiosis in the present sample of examined children was 27.8% by using MZN stain. Youssef *et al.* [21] reported that the prevalence of cryptosporidiosis in Egyptian patients varied significantly from 0% to 47%. This wide range in prevalence could be explained by various causes such as the demographics of the cases, personal habitats,

Table 4. Distribution of other parasitic infections among the 97 examined cases.

Parasite	Positive no.(%)
Giardia lamblia	13 (13.4)
Entamoeba histolytica/dispar	7 (7.2)
Hymenolepis nana	9 (9.3)
Enterobius vermicularis	6 (6.2)
Ascaris lumbricoides	2 (2.1)
Total	37 (38.1)

environmental factors as well as differences in diagnostic procedures [22]. In Cairo, Fathy *et al.* [23] and Abdelrazek *et al.* [24] found that *Cryptosporidium* prevalence was 22.4% and 25.8%, respectively, in children with diarrhea using nested PCR. Higher rates were detected by Helmy *et al.* [25] and Ghoneim *et al.* [26] (49.1% and 33.9%, respectively). In contrast, lower prevalence rates were reported by Sadek [27] and Ahmed *et al.* [28] (10.9% and 17.14% by microscopical examination, respectively).

Worldwide prevalence rates were detected. In Jordan, Latif and Rossle[29] stated that the rate of cryptosporidiosis in diarrheic children was 37.3%. In Pakistan, Khan *et al.* [1] found that positive cases by MZN staining were 29.88% of children aged 3–10 years. In India, *Cryptosporidium* prevalence rate was 22.5% of immunocompetent children [30].

Table 3. Distribution of *Cryptosporidium* genotypes according to demographic data and clinical manifestations.

Variables	C. hominis		C. parvum		χ^2	*p*-value	Odds ratio (95% CI)
	N = 15	%	N = 7	%			
Age group							
1-5 years	8	53.4%	2	28.6%			
6-10 years	5	33.3%	3	42.8%	1.4	0.5	NA
11-15 years	2	13.3%	2	28.6%			
Sex							
Male	9	60.0%	3	42.9%	FET	0.4	0.5 (0.1-3.1)
Female	6	40.0%	4	57.1%			
Residence							
Rural	13	86.7%	7	100.0%	FET	0.3	0.6 (0.4-0.8)
Urban	2	13.3%	0	0.0%			
Animal contact							
NO	6	40.0%	1	14.3%	FET	0.3	0.2 (0.02-2.6)
Yes	9	60.0%	6	85.7%			
Water source							
Tap	12	80.0%	6	85.7%			
Underground	2	13.3%	1	14.3%	0.4	0.7	NA
Filtered	1	6.7%	0	0.0%			
Stool consistency							
Watery	5	33.3%	3	42.8%			
Loose	6	40.0%	2	28.6%	0.3	0.8	NA
Soft	4	26.7%	2	28.6%			
Other manifestations							
Diarrhea only	4	26.7%	4	57.1%			
Abdominal pain	4	26.7%	2	28.6%			
Abdominal pain & fever	3	19.9%	1	14.3%	3.2	0.4	NA
Abdominal pain& vomiting	4	26.7%	0	0.0%			

Only the positive samples by MZN were then subjected to nested PCR-RFLP targeting COWP gene for characterization of *Cryptosporidium* genotypes. The COWP gene is one of the most target genes used in several studies for *Cryptosporidium* detection and genotyping [17,31-32]. Furthermore, Yu *et al.* [35] reported that a high sensitivity was obtained from nested PCR targeting COWP gene, as it allowed detection of a single *Cryptosporidium* oocyst.

On the contrary, Lindergard *et al.* [34] found that COWP primers were less sensitive than the Small subunit ribosomal ribonucleic acid (SSU-rRNA) and thrombospondin-related adhesive protein C2 (TRAP-C2) primers. Also, Ghoshal *et al.* [35] revealed that SSU-rRNA gene was more sensitive (100%) than COWP gene (90%); however, the specificity of both was the same (100%).

In the current work, the predominance of anthroponotic genotype *(C. hominis)* over the zoonotic genotype *(C. parvum)* was in agreement with other studies in Egypt as Helmy *et al.* [25] who revealed that *C. hominis* (60.5%) was the most frequent species isolated. Also, El-Badry *et al.* [36] reported that 89.3% were *C. hominis*. Likewise, several molecular studies revealed the dominance of *C. hominis* in Mexico [37], Lebanon [38], India [35], Gambia [39], and Brazil [40]. However, our results contradict those obtained by Eraky *et al.* [41], who revealed that 82% of infected children in Egypt had *C. parvum* genotype. In Qatar, Boughattas *et al.* [42] reported that genotyping of *Cryptosporidium* spp. showed a predominance of *C. parvum* in 92% of infected children. In Iran, Mahmoudi *et al.* [43] found that all isolates of the species from children were identified as *C. parvum*.

Regarding age distribution of infection, the present study showed that *Cryptosporidium* infection was more frequent among children aged 1-5 years. These results were in agreement with Al-Jawabreh *et al.* [44] and Mahmoudi *et al.* [43], who reported that the highest frequency of cryptosporidiosis was in children under five years. This may be because children in this age group eat without washing their hands, lack knowledge about hygiene and immaturity of gut mucosa [45]. However, Abdelrazek *et al.* [24] revealed that the most significant rate of *Cryptosporidium* infection was noticed in age 5-12 years old.

Our study showed that males were slightly more susceptible to *Cryptosporidium* infection than females (55.6% and 44.4%, respectively), but the difference is statistically insignificant. This higher infection rate among males could be clarified by that males have more chance for outside activities than females, such as swimming in public pools, drinking contaminated well-water, and dealing with farm animals [13]. However, Elshahawy and AbouElenien [22] found that the percentage of infected females was significantly higher than males.

Concerning the distribution of positive cases according to children's residence, results showed that 85.2% of them were from rural areas while only 14.8% were urban inhabitants, with statistically significant difference between them (P=0.004). Hossain *et al.* [39] found that the high percentage among individuals from the rural areas could be due to low socio-economic standards which are associated with unhygienic conditions such as poor food preparations and storage and increased exposure to zoonotic infection. Conversely, Helmy *et al.* (2013) and Gharieb *et al.* [46] stated that no significant associations were noticed between *Cryptosporidium* infection and residence in rural areas.

In the present work, *Cryptosporidium* infection was more common among children with history of animal contact as they were (66.7%) of the total positive cases with statistically significant difference ($p = 0.01$). In the same manner, Hossain *et al.* [39] and Mahmoudi *et al.* [43] revealed a positive correlation between *Cryptosporidium* infection and animal contact. The infected livestock animals (cattle and sheep) have the liability to excrete a large number of *Cryptosporidium* oocysts, contaminate the environment, and infect individuals who live in close proximity to these animals, particularly in rural areas [47].

As regard stool consistency, the highest prevalence of infection was in loose stool specimens (44.5%), followed by watery (33.3%) then soft stool (22.2%) with no statistical significance. This was in agreement with El-Helaly *et al.* [45], who revealed that 44.4% of positive fecal specimens were loose. On the contrary, Squire *et al.* [48] found that most *Cryptosporidium* positive cases had formed stool.

In this study, diarrhea, abdominal pain, vomiting and fever were the main detected signs in examined children. *Cryptosporidium* positive and negative groups did not show significant differences regarding the clinical manifestations. This result was in line with Sadek [27] and Abdel Gawad *et al.* [31]. However, Mohaghegh *et al.* [49] reported a statistically significant association between abdominal pain and *Cryptosporidium* infection. Adamu *et al.* [50] found that vomiting was more common in *Cryptosporidium*-positive than *Cryptosporidium*-negative individuals.

Regarding *Cryptosporidium* genotypes, in our study, *C. parvum* was more common among children

aged 6-10 years (42.8%), but *C. hominis* was more common in 1-5 years age group (53.4%) with no statistical significance. Also, Wielinga *et al.* [51] and Lochlainn *et al.* [52] reported that *C. parvum* was detected at an older age than *C. hominis.* However, Abdelrazek *et al.* [24] found that *Cryptosporidium* genotypes had nearly similar age distribution.

There was no significant association between *Cryptosporidium* genotypes and gender distribution. This was in agreement with Waldron *et al.* [53] and El-Badry *et al.* [36]. Samie *et al.* [54] found that *C. hominis* had equal sex ratio, but *C. parvum* was most familiar among females (75%) than males (25%).

There was no statistically significant difference between the C. parvum and C. hominis positive cases regarding residence and animal contact. Also, Waldron *et al.* [53] found that both species were detected in urban and rural regions. However, Adamu *et al.* [50] reported that history of animal contact was associated with overall *Cryptosporidium* infections, especially with *C. parvum.* This association was mainly due to contact with calves.

There was no statistically significant difference between *C. parvum* and *C. hominis* positive cases regarding clinical manifestations in this work. Conversely, Xiao and Feng [55] detected that the variety of the clinical presentations might be attributed to the diversity of *Cryptosporidium* genotypes. Dey *et al.* [56] found that *C. hominis* infection was frequently accompanied by nausea and vomiting. Insulander *et al.* [57] noticed that individuals infected by *C. parvum* had diarrhea with a longer duration.

Other parasites were also detected during stool analysis. Shalaby and Shalaby [58] reported that 25% of participating children had *Cryptosporidium*, followed by *G. lamblia* (23%), *Cryptosporidium,* and *E. histolytica* (11.7%), and *Cryptosporidium* and *Ascaris* (6.7%). Abdel Gawad *et al.* [31] detected that 5% of examined cases were infected by *Cryptosporidium* and *G. lamblia.*

Conclusions

Cryptosporidium is a common intestinal parasite among children with diarrhea. Both *C. hominis* and *C. parvum* are detected in children. *C. hominis* was relatively more common in children than *C. parvum.* Further studies with larger sample sizes are highly recommended for determining *Cryptosporidium* sub-species with subsequently identifying transmission cycles for better management and control.

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Conflict of interests: No conflict of interests is declared.