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CRYPTOSPORIDIUM HOMINIS PREDOMINANCE AMONG SYMPTOMATIC EGYPTIAN CHILDREN

By
SAMIR A.TAHA[1*], ZEIN ABD AL AAL[1], NESREEN S. SALEH[2] AND AYMAN A. EL-BADRY[3]
Department of Zoology, Faculty of Science, Zagazig University[1], Department of Zoology, Faculty of Science, Benghazi University, Libya[2] and Department of Medical Parasitology, Faculty of Medicine, Cairo University[3], Cairo, Egypt
(*Correspondence: sameertaha@hotmail.com, Mobile 01003811559)

Abstract

Cryptosporidiosis is a worldwide gastrointestinal disease caused by a protozoan parasite; *Cryptosporidium*. This study determined the prevalence of *Cryptosporidium* spp. in fecal samples collected from children, and assessed the association between *Cryptosporidium* in stool with the patient's age, gender and with seasonality. Fecal samples were collected from 176 children (1-12 years) attending gastrointestinal outpatients' clinics in Aboul-Reesh Cairo University Pediatrics Hospital, Egypt. The samples were examined microscopically and by nested polymerase chain reaction (nPCR) assay targeting *Cryptosporidium* oocyst wall protein (COWP) gene. The nPCR products were digested by restriction enzyme *Rsa* I. The obtained fragments were resolved by electrophoresis.PCR was more sensitive than microscopic examination in estimating *Cryptosporidium* prevalence. *Cryptosporidium* oocysts were found in 4.5% (8/176) of samples. *Cryptosporidium* DNA was in 11.9% (21/176). *C. hominis* (17 cases or 81%) was the predominant species among symptomatic children, peaking in the summer and a small rise in autumn. This study demonstrated that age, gender and season might shape the prevalence of such protozoa in a given population.

Keywords: *Cryptosporidium*, children, COWP gene, PCR-RFLP, nPCR, prevalence

Introduction

Cryptosporidium infects a wide range of vertebrates, including humans, recognized as an important gastrointestinal human pathogen (Caccio *et al*, 2005). *Cryptosporidium* spp. are well adapted to zoonotic transmission usually occurs through a direct fecal-oral route and through oocysts contaminating water or food (Smith *et al*, 2007). *Cryptosporidium* spp. can cause a wide spectrum of symptoms, from severe life-threatening diarrhea and vomiting in immunocompromised patients to asymptomatic and self-limiting infection in immunocompetent individuals (Omrani *et al*, 2015). The parasite can be of zoonotic or anthroponotic origin, and has been implicated in diarrhea outbreaks in different parts of the world (Xiao 2010; Adamu *et al*, 2014). *Cryptosporidium* is listed as a neglected disease by the World Health Organization, largely due to a lack of studies in developing countries, but lastly it gained increasing attention (Savioli *et al*, 2006).

Cryptosporidium has 24 valid species and more than 44 genotypes infecting many vertebrates, including animals and humans, which differ significantly in their molecular signatures (Cama *et al*, 2008; Xiao 2010; Ryan *et al*, 2014). There are many intestinal and gastric *Cryptosporidium* species that are capable of establishing infection in immunocompetent and immunocompromised individuals (Akiyoshi *et al*, 2003; Fayer *et al*, 2010; Elwin *et al*, 2012). Among these species, *Cryptosporidium parvum* (which also infects ruminants and other animals) and *C. hominis* (almost exclusively found in humans) are the most common species in clinical human infections (Sulaiman *et al*, 2005).

Using the molecular diagnostics has greatly enhanced the detection of infection and transmission of *Cryptosporidium* (Xiao, 2010). PCR) has been frequently used for identifying the species and genotypes of *Cryptosporidium* due to its higher sensitivity than microscopic examination (Stark *et al*, 2008; Salyer *et al*, 2012).

The study aimed to determine the prevalence of *Cryptosporidium* spp. in fecal samples collected from children, and to assess the association between *Cryptosporidium* in stools with patient's age, gender and with seasonality.

Materials and Methods

The present cross-sectional study was conducted from August 2016 to July 2017 throughout the 4 different seasons. A total of 176 fecal samples were collected from symptomatic Egyptian children from Great Cairo, attending GIT outpatient clinics at Aboul-Reesh hospital Cairo. This study has been approved by the ethical committee of Zagazig University and has been performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or with comparable ethical standards. Families of all children were informed verbally about the purpose of the study, and the collection of samples was performed after obtaining consent. Participation in the study was optional.

Microscopic examination: A single fecal sample from each child was collected and exami-ned after acid-fast staining for *Cryptosporidium* oocysts (El-Naggar *et al*, 2006).

Sample processing for molecular analyses: Molecular analyses were done at the lab of Molecular Medical Parasitology (LMMP), Department of Medical Parasitology, Faculty of Medicine, Cairo University, Egypt. Each collected fecal sample was subjected to *Cryptosporidium* copro-DNA amplification by nPCR and specification of *Cryptosporidium* species using PCR-RFLP.

Total genomic DNA was extracted from the stool samples using the Favor Stool DNA Spin Columns Isolation Mini Kit (Favorgen Biotech Corporation ping-Tung 908, Taiwan) with a modification in the form of prolongation of incubation at the temperature 95 for 1 hour after thermal shock (cycling of deep freezing in liquid nitrogen for 5 min and immediately transferred into a water bath at 95°C for 5 min; repeated for 5 cycles), then the extracted DNA was assessed for concentration and purity using Qubit 2.0 Fluor meter and then stored at -20°C.

The nPCR was carried out for all the collected fecal samples (n= 176) in two consecutive reactions targeting the COWP gene. The first reaction a fragment of 769 base pair (bp) was amplified using a set of primers (Pedraza-Díaz *et al*, 2001). The produced fragment was used as a template in a second reaction in whicha553bp was amplified using inner primers set (Spano *et al*, 1997). Each reaction was done using a PCR mix: 12.5µl PCR Master Mix [Thermo Scientific, UK), 1µl of 200 nmol/l of each forward and reverse primer, 2.5µl of template DNA, 0.1µl Taq polymerase (5U/µl) [product Thermo Scientific]and 7.9µl of sterile distilled water to complete a total volume of 25µl. Reactions were performed in a gradient thermal cycler (thermo-cycler, Biometra; Applied Biosystems, California, USA) after adjusting the thermal profile to initial denaturation at 95°C for 4 min, followed by 30 cycles of amplification, each consisting of denaturation at 94°C for 60 seconds, annealing at 65°C for 60 second and extension at 72°C for 60 second. Final elongation was performed at 72°C for 10 min. The second-round PCR was identical to the first-round PCR except for denaturation at 94°C for 50 second, annealing at 54°C for 30 second, and extension at 72°C for 50 second. The amplified PCR products were separated by electrophoresis on 2% agarose gel and further visualized under a UV trans-illuminator after being stained with ethidium bromide (Tab. 1).

Cryptosporidium genotyping by PCR-Restriction fragment length polymorphism (PCR-RFLP): A volume of 10µl of nPCR product was digested in a mixture consisting of 1µl *Rsa*I (No. ER1121, Thermo Scientific) 2µl green buffer, and17µl nuclease-free water to reach a volume of 30µl. The mix was mixed gently followed by spinning down for a few seconds and then incubated at 37°C for 5min. *Cryptosporidium* geno-

types were detected by using electrophoresis in 3.2% typing-grade agarose gels containing ethidium bromide, and gels were visualized under UV trans-illumination. Digestion pattern of the fragment was different for each characteristic *Cryptosporidium* species infecting human: *C. hominis* (34,106,125 & 285bp) and *C. parvum* (34,106 and 410bp).

Statistical analysis: Data were analysed a chi-square test of association on the infected and non-infected samples for all risk factors. Significance of association was expressed as *P*-value according to the following parameters: P >0.05: non-significant, P <0.05: significant. The statistical analyses were conducted using Graph Pad Prism V.5software (Graph Pad Software, Inc., San Diego, California).

Results

The patients' gender distribution was 92 males & 84 females. Ages ranged from 1 to 12 years old; 25 were 1 to 2 years, 87 between 3 to 8 years and 64 between 9 to 12 years.

Microscopically the oocysts in children's feces were confirmed morphologically to be those of *Cryptosporidium*. Oocysts were spherical in shape and had smooth wall. Out of the 176 microscopically examined fecal samples, 8 (4.5%) fecal samples were *Cryptosporidium* oocyst positive (Tab. 2).

The nPCR analysis that targeted the COWP gene (553bp) identified *Cryptosporidium* DNA in 11.9 % (21/176) of the samples. With respect to gender, there was higher prevalence of infection in samples collected from male children than that from female children (Fig. 1). The higher prevalence was observed for children in early childhood (3-8years) than children in late childhood (9-12 years). No positive cases were found in infants (0-2 years). Also, *Cryptosporidium* was more frequently in summer and autumn and less in spring and winter (Tab. 3).

Using Chi-Square test of association, there was significant association between being infected with *Cryptosporidium* and the age, gender and season in which the samples were collected (P = 0.04) using nPCR.

C. hominis pattern was shown 17 (81%) of digested *Cryptosporidium* DNA, while 4 (19%) showed *C. parvum* pattern (Fig. 2).

Table 1: Primers used for the PCR-based genotyping of *Cryptosporidium*

Primers	Primer Sequence	Size (bp)	PCRs	Reference
BCOWP F	5' ACCGCTTCTCAACAACCATCTTGTCCTC 3'	769 bp	1st PCR	Pedraza-Díaz *et al*, 2001
BCOWP R	5' CGCACCTGTTCCCACTCAATGTAAACCC 3'			
Cry-15	5' GTAGATAATGGAAGAGATTGTG 3'	553 bp	2st PCR	Spano *et al*, 1997
Cry- 9	5'GGACTGAAATACAGGCATTATCTTG3'			

Table 2: *Cryptosporidium* spp., in samples (n=176) using stained microscopic samples and nPCR.

Technique	No. examined	No. infected	Prevalence (%)
Microscopic examination	176	8	4.5
nPCRs	176	21	11.9

Table 3: *Cryptosporidium* in different children gender, age and in different seasons by microscopy and nPCR.

Variants		No. examined	Microscopy		nPCR		*P value*
			No.	(%)	No.	(%)	
Sex	Male	92	6	6.52	16	17.39	0.045 (*)
	Female	84	2	2.38	5	5.95	
Age	Infant (1-2 years)	25	0	0	0	0	0.045 (*)
	Early childhood (3-8 years)	87	5	5.74	17	19.54	
	Late childhood (9-12 years)	64	3	4.68	4	6.25	
Season	Summer	72	5	6.94	11	15.27	0.045 (*)
	Autumn	33	2	6.60	6	12.12	
	Winter	21	0	0	1	4.76	
	Spring	50	1	2	3	6	

Discussion

The *Cryptosporidium* infection prevalence in the Egyptian patients varied significantly from 0% to 47% (Youssef *et al*, 2008).

In the present study, 4.5% prevalence of *Cryptosporidium* spp. was microscopically identified, which was lower than that measured by using the nPCR approach (11.9%). This agreed with reports in Egyptian children in Cairo. El-Helaly *et al*. (2012) and El-Matrawy *et al*. (2017) detected *Cryptosporidium* oocysts in 2.6%, 1.3%, respectively, using microscopy after staining with AF stain. In contrast, higher prevalence (32%) of cryptosporidiosis was reported by Al-Braiken *et al*. (2003) in Saudi Arabia using microscopy. In Egypt, El-Hamshary *et al*. (2008); El-Settawy and Fathy (2012) reported relatively high prevalence rates in Egyptian children, 21% & 18.6%, respectively, using the same technique. These data gave discrepancies in the prevalence of *Cryptosporidium*, and suggested the need for more accurate and sensitive methods.

In the present study, using nPCR showed that 11.9% of children had *Cryptosporidium* infection. This was higher than those of Abd-El-Kader *et al*. (2012); Sharma *et al*. (2013) and El-Matrawy *et al*. (2017) who reported cryptosporidiosis among diarrheic Egyptian children using nPCR to be 4.6%, 3.8% & 6%, respectively. On the other hand, among diarrheic Egyptian children using nPCR El-Hamshary *et al*. (2008); Abdelrazek *et al*. (2016); El-Badry *et al*. (2017) and Salyer *et al*. (2012) found that the prevalence of *Cryptosporidium* was relatively higher than that in the present study (25%, 25%, 23.6%, & 32.4%) respectively..

The discrepancies in the reported prevalence of other studies and the current one might be attributed to differences in population demographics, environmental, behavioral and socioeconomic factors, as well as differences in timing of sample collection (e.g. summer vs. winter).

Age and gender proved to be risk factors affecting the prevalence of *Cryptosporidium* in certain populations (Abdelrazek *et al*, 2016).

In the present study, the *Cryptosporidium* prevalence was found in early and late childhood, but not in infants. These results agreed with studies conducted by Abdel-Messih *et al*. (2005); Al-Shamiri *et al*. (2010); Iqbal *et al*.(2011) and Abdelrazek *et al*.(2016). In contrast, El-Helalya *et al*. (2012) found that the peak prevalence of cryptosporidiosis was in children aged 1-5 years old. All these factors might increase probability of *Cryptosporidium* detection or exposure to infection (Abdelrazek *et al*, 2016).

In the present study, *Cryptosporidium* was more common among males than females, which could be due to a larger sample size of males than females, or might be due to more frequent exposure of male children to gardens and farm animals. This agreed with previous studies (Abd El-Kader *et al*, 2012; El-Badry *et al*, 2015; Ibrahim *et al*, 2016). On the other hand, Yoder *et al*. (2007) found that most of the reported cases in 2005 occurred among females, and Mathew *et al*. (2014) also reported higher cryptosporidiosis infection in females (57.1%) compared to males (42.9%). El-Helalya *et al*. (2012), Abdelrazek *et al*. (2016) showed that males and females had similar positive rates without significant association. Similarly, Abd El Kader *et al*. (2012) found that infection rates did not vary with gender.

The present study revealed that the prevalence of *Cryptosporidium* peaked in summer with, small a rise increase in autumn. These results agreed with El-Badry *et al*. (2015) who found that *Cryptosporidium* was more prevalent in summer and a small rise in spring. In contrast, Iqbal *et al*. (2011) found that the maximum cryptosporidiosis cases were detected in winter.

In the present study, two genotypes, *C. hominis* and *C. parvum*, were detected in stool of the children, with *C. hominis* predominance in all patient groups. Similarly, other studies in Egypt (El-Badry *et al*, 2015; Abdelrazek *et al*, 2016; Abdelrazek *et al*,

2016; Ghallab *et al*, 2016; El-Badry *et al*, 2017), as well as worldwide studies (Bushen *et al*, 2007; Xiao, 2010) reported *C. hominis* predominance.

Conclusion

The conventional microscopic technique used for the diagnosis of *Cryptosporidium* has a limited capacity and low sensitivity in identifying *Cryptosporidium* spp. On the other hand, nPCR RFLP analysis of COWP gene fragment proved to be of high sensitivity and was useful for diagnosis and genotyping of *Cryptosporidium*. The *C. hominis* was more prevalent in stool samples of symptomatic Egyptian children living in Great Cairo. This suggests that *C. hominis* is of relatively greater risk for humans infections than other species, notably *C. parvum*. The infection rate was higher in male than female children and mainly in early and late childhood. The rate of infection was higher in summer and autumn than in spring and winter months. This study showed that certain risk factors might determine the prevalence of such protozoa in a given population.

Conflict of interest: The authors declare that they neither have conflict of interest nor received fund.

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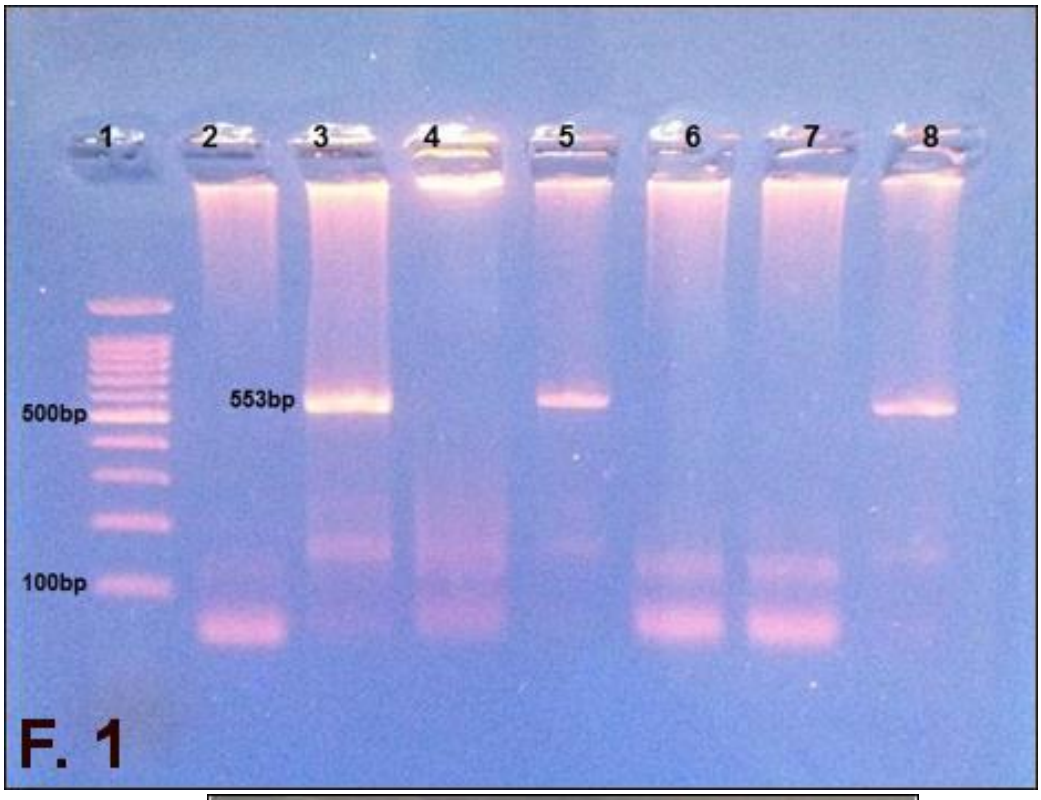
Explanation of figurers

Fig. 1: Agarose gel electrophoresis for the products of the nPCR targeting COWP gene of *Cryptosporidium* at 553 bp. Lane1: DNA molecular weight marker100 bp; lane2: negative control; lane3: positive control; lane 4, 6 and 7: negative samples; lane 5 and 8: positive samples.

Fig. 2: Agarose gel electrophoresis showing RFLP products after digestion with *Rsa* I enzyme endonuclease with *Cryptosporidium hominis*. Lane1: DNA molecular weight marker100 bp; lane 2 and 3: undigest product of the nested polymerase chain reaction (nPCR) targeting the COWP gene of *Cryptosporidium* at 553 bp; lane 4 and 5: *Cryptosporidium hominis* genotype digestion product at 34, 106, 125 and 285 bps (34 band is very small, faint and difficult to see).


1
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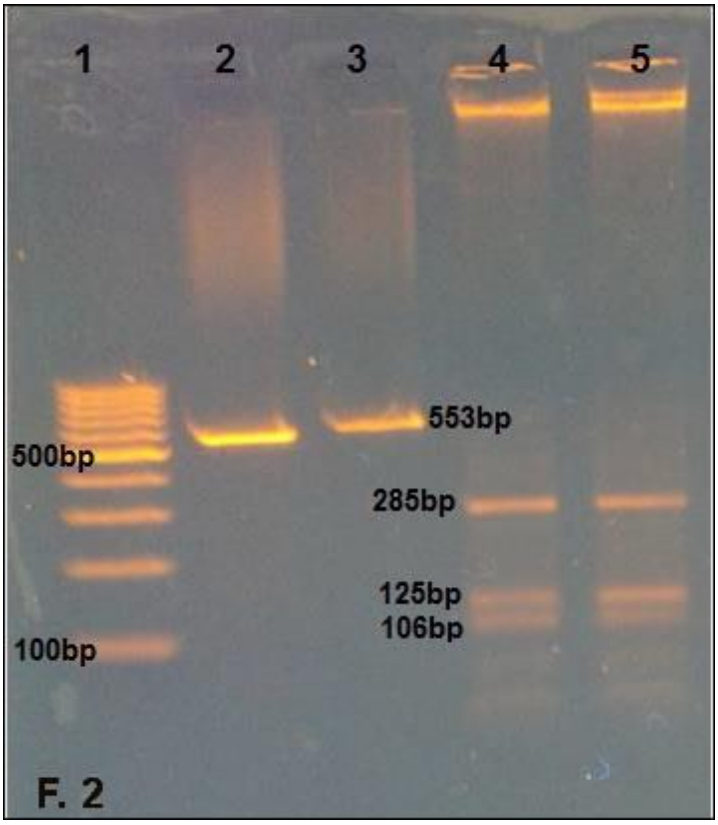
553bp
100bp

F. 1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F. 2