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ORIGINAL ARTICLE

Cryptosporidium genotypes and associated risk factors in a cohort of Egyptian children

Ayman A. El-Badry[1] · **Inas Z. Abdel Aziz**[2] · **Eman Y. Shoeib**[2] · **Marwa M.I. Ghallab**[2,3]

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Abstract *Cryptosporidium* is one of the most common, worldwide diarrheal diseases caused by parasites. Due to absence of an effective treatment, determining the prevailing species of *Cryptosporidium* is a key in identifying its transmission dynamics and a necessary precursor required for the planning and implementation of effective preventive and control strategies. This PCR-RFLP study was done to determine the prevalence of *Cryptosporidium* species in the stool of a cohort of Egyptian children and evaluate/assess associated risk factors for susceptibility to cryptosporidiosis, due to the lack of existent studies addressing *Cryptosporidium* transmission dynamics in humans and assessed risk factors in Egypt. Stool samples were collected from 431 children; 331 diarrheic and 100 apparently healthy non-diarrheic children; their data were recorded. Samples were processed for Copro-nPCR targeting Hsp90 gene and PCR-RFLP analysis for species identification. Variables which showed statistical significance for *Cryptosporidium* were included in a logistic regression analysis to identify the estimated risk. Out of 84 (19.5%) *Cryptosporidium*-positive samples (78 diarrheic and 6 non-diarreic), 75 (89.3%) were *Cryptosporidium hominis*, 6 (7.1%) were *Cryptosporidium parvum*, and 3 (3.6%) were non-typed. There was a significant association between *Cryptosporidium* detection in stool and the estimated risk factors: diarrhea, soft stool, and drinking from tap water. *Cryptosporidium* is an indigenous, prevailing intestinal parasite among children in Cairo that physicians must consider, especially in diarrheic, preschool-aged children, who drink from tap water. The finding of a predominance of *C. hominis* indicates anthroponotic rather than zoonotic transmission.

Keywords *Cryptosporidium* · Copro-nPCR · RFLP · Hsp90 · Estimated risk factors

✉ Ayman A. El-Badry
aelbadry@kasralainy.edu.eg; aaelbadry@uod.edu.sa

[1] Department of Microbiology—Medical Parasitology Section, College of Medicine, University of Dammam, P.O. Box: 2114, Dammam 31451, Saudi Arabia

[2] Medical Parasitology Department, Kasr Al-Ainy Faculty of Medicine, Cairo University, Giza, Egypt

[3] Medical Parasitology, Kafr El-Sheikh Faculty of Medicine, Kafr El-Sheikh University, Kafr El-Sheikh, Egypt

Introduction

Cryptosporidium possesses 24 valid species and more than 44 genotypes infecting many vertebrates; including humans and animals, which differ significantly in their molecular signatures (Cama et al. 2008). Thirteen cases of intestinal and gastric *Cryptosporidium* species infecting immunocompetent and immunocompromised humans have been reported until now (*C. parvum*, *C. hominis*, *C. meleagridis*, *C. felis*, *C. canis*, *C. suis*, *C. muris*, *C. andersoni*, *C. fayeri*, *C. cuniculus*, *C. ubiquitum*, and *C. viatorum*) (Fayer et al. 2010; Elwin et al. 2012). Among them, *C. parvum* (which infects not only humans but also ruminants and perhaps a few other animals) and *C. hominis* (which is almost exclusively a human parasite) are the most common species involved in clinical infections (Sulaiman et al. 2005).

Cryptosporidium is listed as a neglected disease by the World Health Organization, largely due to a lack of studies in developing countries, but is now gaining increasing attention (Savioli et al. 2006).

Because of the limitations in the specific detection of *Cryptosporidium* using microscopic, immunological, and/or

flowcytometric methods, a wide range of nucleic acid-based methods had been developed and evaluated for the identification of species, as well as the detection of genetic variation within and among species of *Cryptosporidium* with a high diagnostic yield (Smith et al. 2006).

The current study aimed to determine the prevalence of *Cryptosporidium* among a cohort of Egyptian children targeting the Hsp90 gene and RFLP analysis for species identification. Due to the lack of existent studies assessing risk factors in Egypt, this study was designed to investigate the role of collected data variables for susceptibility to *Cryptosporidium* infection among individuals.

Methods

Sampling and data collection

A cross-sectional study was designed involving 431 stool samples (331 diarrheic and 100 apparently healthy non-diarrheic children) collected from children of both sexes ranging in age from 1 to 12 years, presenting with diarrhea and/or other GIT symptoms recruited from the outpatient clinic in Abu El Rish pediatric hospital, Kasr Al-Ainy School of Medicine, and Cairo University from April 2013 to January 2014. Data included the recording of their sociodemographic and environmental data. Parents of young children provided consent forms and responded to the questionnaire.

Extraction of the genomic DNA

Genomic DNA was extracted using Favor Prep stool DNA isolation Mini Kit (Favorgen Biotech corporation ping-Tung 908, Taiwan, Cat. No. FASTI001) with modification in the form of prolongation of incubation to 95 °C for 1 h after thermal shock (cycling of deep freezing in liquid nitrogen for 5 min and immediately transferred into a water bath 95 °C for 5 min; repeated for 5 cycles) then the purified DNA was measured for concentration and purity.

Copro-nPCR/RFLP analysis

The nPCR was done by analysis of Hsp90 gene. Amplification of 835–844 for the primary reaction and a fragment of 676–685 for the secondary reaction, using two sets of oligonuclutide primers (Table 1) with the reaction components and the cycling conditions carried out according to Feng et al. (2009) with modification in the form of 12.5 μl master mix, 200 nM from each primer, and 3 μl of the template DNA for the primary reaction and 1 μl for the secondary one in a total volume of 25 μl and 50 °C annealing temperature for the primary and the secondary reactions. The amplified products were visualized with 1.5% agarose gel electrophoresis after ethidium bromide staining. The amplified products of nPCR of positive samples were digested using *StyI* and *HphI* endonuclease (Table 2) according to manufacturer's instruction and resolved, using 3% metaphor electrophoresis after ethidium bromide staining.

Table 1 The used primers and their sequences (Feng et al. 2009)

	Primers	Sequence	Expected product size (bp)
1ry PCR	HSp90 f3	5́-CTA GTG AAA GCT ACG AGT TCC AA-3	835–844 bp[a]
	HSp90R3	5́-TCT ATTTCA CCT TCG GCG GAA AA-3	
nPCR	HSp90 f4	5́-GGA TAT TAT TAT TAA CTC TCT CTA TTC TC GAA-3	676–685 bp[b]
	HSp90R4	5́-CCA TAT TGC CTT TTC TAC ATT AAC-3	

[a] 835 bp for *C. parvum* and *C. hominis* and 844 bp for *C. muris*

[b] 676 bp for *C. parvum* and *C. hominis* and 685 bp for *C. muris*

Statistical analysis

Data were statistically analyzed using the statistical package SPSS version 17 (Chicago, IL, USA). Data were analyzed with Fisher's exact test and multiple logistic regression. All variables that were significantly associated with the *Cryptosporidium* prevalence in the univariate model were included in a multivariate logistic regression.

Results

Out of 431 examined stool samples with nPCR, 84 (19.5%) stool samples were *Cryptosporidium* positive (78 diarrheic and 6 non-diarrheic); among them 75 (89.3%) were *C. hominis*, 6 (7.1%) were *C. parvum*, and 3 (3.6%) were non-typed. Their relative data were tabulated in Table 3.

Among the studied variables, only diarrhea, the type of water, and stool consistency were significantly associated ($P<0.05$) with detection of *Cryptosporidium*. After these variables were subjected to a multivariate analysis using logistic regression, there was an estimated increase in the risk of *Cryptosporidium* among children suffering from diarrhea 4.8 times greater than the non-diarrheic children, with soft and liquid stools, 6.5 and 3.3 times, respectively, relative to formed stool, and 4.4 times for children drinking tap water relative to mineral water (Table 4).

In contrast, none of the studied variables, including gender distribution, age distribution, animal contact habits, and gross

Table 2 Hsp90 RFLP (restriction enzymes) (Feng et al. 2009)

Species		Fragment(s) (bp)			
		PCR product	StyI digestion	HphI digestion	BbsI digestion
Intestinal	*C. hominis*	676	42, 634	80, 135, 461	676
	C. parvum	676	42, 119, 515	46, 215, 415	676
	C. meleagridis	676	42, 119, 155, 360	215, 461	676
	C. canis	670	230, 440	135, 165, 370	670
	Cervine genotype	676	208, 230, 239	135, 541	670
	C. suis	673	111, 119, 443	43, 630	676
Gastric	*C. andersoni*	685	685	685	32, 87, 584
	C. muris	685	685	685	32, 653

features of stool showed any significant association among genotypes ($P > 0.05$) (Table 4).

Discussion

Cryptosporidium is a highly prevailing parasite among studied diarrheic children for about a quarter of them (23.6%) using nPCR, with an overall prevalence of 19.5% in both groups. There is a difference in the reported prevalence of *Cryptosporidium* in Egypt in the last decades; most of the studies report a high molecular prevalence of up to a quarter of examined patients (El-Settawy and Fathy 2012; Fathy et al. 2014; Ghallab et al. 2016), however, much lower results (4.6%) were also reported in Egypt using PCR (Abd El-Kader et al. 2011) and these studies claimed that the presence of fecal inhibitors and contaminants such as bilirubin, bile salts, and others can inhibit DNA amplification, yielding less accurate detection results (Abd El-Kader et al. 2011).

Until now, few studies in Egypt have addressed the *Cryptosporidium* transmission dynamics in humans, despite it being pivotal in terms of establishing effective prevention and disease control policies. In the present study, *C. hominis*, an anthroponotic *Cryptosporidium* species, was significantly more prevalent (89.3%) than *C. parvum* (7.1%). Mixed infections were not detected, indicating that the main source of *Cryptosporidium* infection in the study group was of human rather than zoonotic source. Akiyoshi et al. (2003) reported that in mixed infections, *C. parvum* predominates and rapidly displaces *C. hominis* which adds more proof that in our study, it is a *C. hominis* predominance and may explain the non-typed species we obtained. Our results are in concordance with those of only one study (Abd El-Kader et al. 2011) and contradictory to those of other Egyptian studies and most studies from other Middle Eastern countries (Eida et al. 2009; Al-Brikan et al. 2008; Hijjawi et al. 2010; Iqbal et al. 2011). Our data and the data of Abd El-Kader et al. (2011) suggest a clear anthroponotic transmission; this discrepancy in the results may be explained by the fact that Abd El-Kader

Table 3 Data of Hsp90 positive cases

			Frequency			*P* value*
			+ve	−ve	Percent	
Group		Symptomatic	78	253	23.6	0.0001*
		Asymptomatic	6	94	6	
Gender		Male	43	184	18.9	0.67
		Female	41	163	25.1	
Age group		Infant	33	101	24.6	0.16
		Early childhood	42	193	17.8	
		Late childhood	9	53	14.5	
Type of water		Tape	75	337	18.2	0.007*
		Filtered	6	6	50	
		Mineral	3	4	42.8	
Animal contact		Yes	27	111	24.3	0.98
		No	57	236	19.5	
Stool	Contents	Mucous Yes	8	45	15.1	0.56
		Mucous No	76	302	20.1	
		Pus >5	35	136	20.4	0.63
		Pus 0–5	49	211	18.8	
	Consistency	Liquid	47	181	18.5	0.0001*
		Soft	31	72	30.08	
		Formed	6	94	6	

Data presented as *n*

**P* value <0.05 is significant

Table 4 Multivariate analysis for nPCR *Cryptosporidium*-positive cases

		OR	95% CI	*P* value*
Group	Symptomatic/asymptomatic	4.8	(2.04–11.5)	0.001*
Type of water	Tap water/mineral	4.4	(1.4–14.3)	0.011*
	Filter/mineral	3.3	(0.7–15.3)	0.117
Type of stool	Liquid/formed	4.06	(1. 7–9.9)	0.001*
	Soft/formed	6.7	(2.7–17.0)	0.002*

Data presented as *n*

**P* value for OR < 0.05 is significant

et al. (2011) and our study were conducted in Great Cairo, while the contrasting results were found in more rural parts of Egypt.

This predominance of *C. hominis* is in accordance with molecular studies in Australia, Canada, Japan, USA, and developing countries (Xiao and Fayer 2008). In contrast, studies in the UK have shown *C. parvum* predominance (McLauchlin et al. 1999, 2000).

Among studied variables, diarrhea, the type of water, and stool consistency showed statistically significant association with cryptosporidiosis. Our results were in agreement with those of other reports in Mexico and Iran, in which there were no reported significant differences between cases with cryptosporidial diarrhea and age, race, ethnicity, or sex (Nair et al. 2008; Saneian et al. 2010; Bushen et al. 2007). It was reported that *C. hominis* is contracted at an earlier age than *C. parvum*. Samie et al. (2006) reported that *C. hominis* was equally distributed between males and females. GIT symptoms, including diarrhea, abdominal pain, flatulence, itching, vomiting, and/or appetite loss (Chauret et al. 1999), animal contact (Hijjawi et al. 2010), and type of water supply (Goh et al. 2004; Beach 2008; Garvey and McKeown 2009; Al-Warid et al. 2012) are well-known risk factors for cryptosporidiosis.

The occurrence of clinical manifestations in *Cryptosporidium* can be related in part to the different *Cryptosporidium* species and subtypes of *C. hominis*. Cama et al. (2008) reported that *C. hominis* was associated with diarrhea, vomiting, nausea, and general malaise, while *C. parvum*, *C. canis*, *C. felis*, and *C. meleagridis* were associated with diarrhea only. Environmental, clinical, and host behavioral factors may act as important risk factors for *Cryptosporidium* infection, susceptibility, or disease prevalence, but do not affect the pathogenicity or the course of the disease (Mumtaz et al. 2010).

In the present study, besides the studied variables that may induce susceptibility of cryptosporidiosis in Egypt, there are added variables such as higher population densities with a greater chance of person-to-person transmission and variation in socioeconomic status within the same geographical areas. This might also explain the discrepancies in the reported results for the prevalence of the disease between past studies and our current research findings.

Conclusion *Cryptosporidium* is an indigenous, prevailing intestinal parasite among children in Cairo with a predominance of *C.hominis*, suggesting anthroponotic transmission rather than zoonotic. The significant majority of *Cryptosporidium* infections were detected in diarrheic patients, who relied upon tap water sources; their stool was soft and yielded more parasites than the patients with liquid stool. Due to the absence of any effective curative treatments, determining the prevailing species of *Cryptosporidium* and identifying the significant etiological risk factors are essential in order to identify its transmission dynamics and plan preventive and control strategies.

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Compliance with ethical standards

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Ethical approval This study was ethically approved by the "Research Ethical Committee," Deanship of postgraduate education & scientific research, Faculty of Medicine, Cairo University in compliance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from patients or their relatives and parents of young children before they responded to the questionnaire.

Conflict of interest The authors declare that they have no conflict of interest.

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