Original Article

Prevalence of *Cryptosporidium* species among immunocompetent and immunocompromised Egyptian children: comparative study

Aliaa M Elsawey[1], Suzan H Elgendy[1], Salama A Abdel-Magied[1], Yousef Mosaad[2], Nairmen Nabih[1]

Departments of Medical Parasitology[1] and Clinical Pathology[2], Faculty of Medicine, Mansoura University

ABSTRACT

Background: *Cryptosporidium* is a protozoan parasite that causes gastrointestinal infection. Each cryptosporidial species usually inhabits a particular host; however cross-infectivity could occur.

Objectives: The aim of the study was to identify prevalent *Cryptosporidium* species among a sample of immunocompetent and immunocompromised children.

Subjects and Methods: This study included 150 children divided as 50 control group (apparently healthy with no complaints) and 100 patients (immunocompetent and immunocompromised) with gastrointestinal manifestations. Stool samples were collected and examined microscopically using modified Zeihl-Neelsen stain (mZN), ELISA for coproantigen and nested PCR (nPCR). Restriction fragment length polymorphism (RFLP) technique was done for species identification of PCR products.

Results: Among the 100 patients, cryptosporidiosis prevalence was 34%, 46% and 59% as detected by mZN, ELISA and nPCR, respectively. Infection was more prevalent in immunocompromised group (84%) than immunocompetent group (34%) as detected by nPCR (P<0.001). Cryptosporidiosis was found to be significantly associated with nausea (P=0.002) and with diarrhea (P=0.04). Detected species among studied children were *C. hominis* (52.5%), *C. parvum* (33.9%), *C. meleagridis* (8.5%) and *C. felis* (5.1%). *C. hominis* was the prevalent species in both immunocompetent and immunocompromised groups.

Conclusion: *C. hominis* was proven to be the more prevalent species among cryptosporidiosis positive children in this study. More attention should be paid to this emerging parasitic infection especially in immunocompromised children.

Keywords: *Cryptosporidium* spp., Egypt, nested PCR, prevalence, RFLP.

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Corresponding Author: Aliaa Elsawey, **Tel.:** +20 1008874025, **E-mail:** aliaaelsawey@gmail.com

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INTRODUCTION

Cryptosporidium is a protozoan inhabitant of the brush borders of the gastrointestinal epithelial cells. It was thought to be a zoonotic pathogen; however, anthroponotic transmission was also established[1]. Infection results from consumption of contaminated water or food including raw milk[2]. Generally, cryptosporidiosis is a short-term sickness in the form of diarrhea and weight loss in immunocompetent children and adults. However, in immunocompromised individuals, infection could be prolonged and life-threatening[3]. A variable prevalence range of 6% to 49% was recorded in different reports on the infection in Egyptian children[4-8].

Molecular techniques for differentiation of species by detection of the small subunit ribosomal ribonucleic acid (SSU rRNA) of *Cryptosporidium* oocyst wall protein genes[9] were reviewed. *C. hominis* and *C. parvum* were recorded as the major species infecting human population. Other species, like *C. felis*, *C. meleagridis*, *C. muris*, *C. canis* and *C. suis* were reported less frequently. Infection with *C. hominis* was linked to different manifestations as diarrhea, nausea, vomiting, general malaise, while infection with *C. parvum* was linked mainly to cases with diarrhea[10,11]. El-Hamshary *et al*.[4], in an early study on immunocompromised patients of different ages from attendants of Suez Canal University Hospitals in Egypt, used multiplex allele specific polymerase chain reaction and recorded 68.4% infection with *C. parvum* and 26.3% with *C. hominis*. Moreover, *C. parvum* was found to be more prevalent (61.5%) in rural localities while *C. hominis* was more prevalent in urban areas (60%). In another attempt to genotypically characterize *Cryptosporidium* spp. in a sample of isolates from calves and children suffering from diarrhea, 88.9% from the children's positive samples were identified as *C. hominis* (*C. parvum* genotype 1) and genotype 2 *C. parvum* was apparently more prevalent in the sample of calves[12].

Our study was conducted to identify the prevalent *Cryptosporidium* species among immunocompetent and immunocompromised children attending Mansoura University Children's Hospital in Egypt.

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SUBJECTS AND METHODS

Subjects: The present case-control study was conducted in laboratories of Mansoura Faculty of Medicine, Egypt during the period from April 2016 to September 2017. One hundred and fifty children aged from 2 to 17 years were included. One hundred children were patients attending different departments of Mansoura University Children's Hospital. They were divided into two groups (each of 50) to include (1) immunocompetent children complaining of gastrointestinal manifestations, (2) immunocompromised children due to chemotherapy for hematopoietic and lymphatic tumors and complaining of gastrointestinal manifestations. In addition, 50 children included as control group were selected from healthy visitors or were accompanying patients attending the hospital for other complaints. All children were subjected to a questionnaire concerning their medical history, and clinical examination.

Sample collection and microscopic examination: Stool samples were collected (from all participated children) in clean, dry, labeled plastic containers and divided into three parts; first part for concentration by formalin-ether technique, followed by mZN[13], and microscopic examination for oocysts. The second and third parts, without any additives, were stored at -20°C for coproantigen detection and molecular processing, respectively.

ELISA technique for coproantigen: ELISA technique was performed for detection of *Cryptosporidium* coproantigen (Epitope Diagnostic's, Inc., San Diego, USA) according to the manufacturer's instructions[14].

Nested PCR: DNA was extracted from frozen fecal specimens using QIAamp DNA stool mini kit (Qiagen, Biocompare, USA) according to the manufacturer's protocols[15,16]. nPCR technique was carried out to amplify SSU rRNA gene of *Cryptosporidium*[17,18]. This technique amplifies a ~830-bp fragment of the SSU rRNA gene by nPCR and differentiates *Cryptosporidium* species by banding patterns in restriction analysis of the secondary PCR products with the enzymes SspI and VspI. The primary PCR and secondary PCR reactions were followed by running 20 µL of the PCR product for electrophoresis on a 1.5% agarose gel[18]. The primers (Bio Basic Canada Inc., Markham, ON, Canada) are listed in table (1).

Table 1. Primers used in the nPCR reactions

Primary primers	**Forward (F1)**	5'-TTCTAGAGCTAATACATGCG-3'
	Reverse (R1	5'-CCCATTTCCTTCGAAACAGGA-3'
Secondary primers	**Forward (F1)**	5'-GGAAGGGTTGTATTTATTAGATAAAG-3'
	Reverse (R1	5'-CTCATAAGGTGCTGAAGGAGTA-3

RFLP technique: Restriction enzymes used were SspI (Enzynomics co. Ltd, Daejeon, Republic of Korea) and VspI (Thermo Fisher Scientific, Massachusetts, USA). Electrophoresis was run on agarose gel with restriction digestion reaction whole volume of 40 µL, and *Cryptosporidium* species were identified based on RFLP banding patterns. Species diagnosis was made by digesting the secondary PCR product (826 to 864 bp) with SspI, and VspI according to Xiao *et al*.[18].

Statistical analysis: Analysis of data was done using IBM SPSS software package version 20.0 (IBM, Chicago, IL, USA). Categorical variables were analyzed by Kruskal-Wallis H and Mann–Whitney U tests. Chi Square test was used when appropriate. P value < 0.05 was considered statistically significant at confidence interval 95%. Logistic regression analysis was performed for the association of cryptosporidiosis as a risk factor for the occurrence of gastrointestinal symptoms.

Ethical approval: Informed consent was obtained from the parents of all participated children. All performed procedures were in accordance with Helsinki declaration. Ethics Review Committee and the Institutional Review Board of the Faculty of Medicine, Mansoura University, Egypt, approved this study with code number R/15.06.26. Infected children were referred to pediatric staff for appropriate treatment with antiprotozoal agent.

RESULTS

Among studied children with gastrointestinal manifestations (n=100), 34% harbored *Cryptosporidium* oocysts, identified by mZN stain (Table 2). Using ELISA, 46% were positive while by nPCR, 59% were positive (Fig. 1). Among control children (n=50), the recorded prevalence was 4%, 6% and 10% as detected by mZN stain, ELISA and nPCR respectively. Statistically relevant cryptosporidiosis (P<0.001) was more prevalent in immunocompromised group (50%, 66% and 84% respectively) than immunocompetent group (18%, 26% and 34%, respectively) (Table 2).

Infection was significantly higher in children aged less than 5 years old (P<0.001). No difference was recorded regarding gender or between urban and rural areas. Gastrointestinal manifestations among studied cases (n=100) showed that nausea and diarrhea were significantly high in *Cryptosporidium* infected group (P< 0.05). *Cryptosporidium* spp. was detected in 31/49 diarrheic children (63.3%). By Likelihood ratio tests among different symptoms, nausea and diarrhea were significantly related to cryptosporidiosis, P=0.002 and 0.04 respectively (Table 3). Cryptosporidiosis was a potential risk factor for nausea in children, with an odds ratio (OR) =3.9 (95% CI = 1.6 to 9.6 and P< 0.01); and also for diarrhea in children, OR =2.3 (95% CI= 1.02 to 5.22 and P< 0.05).

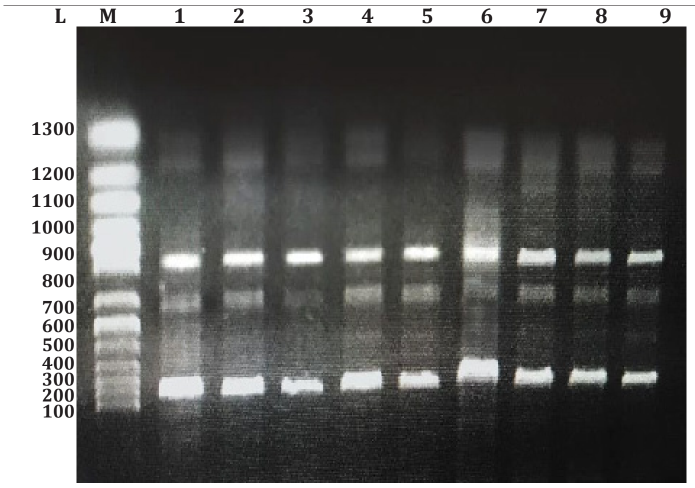

Fig. 1 Diagnosis of cryptosporidiosis by nPCR procedure based on detection of the 830-bp SSU rRNA gene.
M, Molecular markers of 100-bp ladders.
Lane 1-9, Amplified ~830-bp of 18s rRNA gene for *Cryptosporidium*.

Table 2. Prevalence of *Cryptosporidium* infection among controls and patients using mZN, ELISA and nPCR techniques.

Group	Method used		
	mZN Microscopy	**ELIZA**	**nPCR**
	No. (%)	**No. (%)**	**No. (%)**
Control group (n=50)	2 (4)	3 (6)	5 (10)
Patients group (n=100)	34 (34)*	46 (46)*	59 (59)*
Immunocompetent patients (n=50)	9 (18)	13 (26)	17 (34)
Immunocompromised patients (n=50)	25 (50)**	33 (66)*	42 (84)*

*$P< 0.001$, **$P= 0.001$, **mZN:** modified Zeihl Neelsen, **nPCR:** nested PCR

Table 3. Likelihood ratio results of symptoms among patient groups in relation to cryptosporidiosis (n=100).

Symptoms	Likelihood ratio tests	
	Chi-Square	**Significance (*P* value)**
Nausea	9.808	0.002*
Vomiting	2.500	0.114
Diarrhea	4.136	0.042*
Abdominal pain	0.268	0.605
Weight loss	2.925	0.087

*$P< 0.05$

Among samples proved positive by nPCR, RFLP identified *C. hominis* (52.5%), *C. parvum* (33.9%), *C. meleagridis* (8.5%) and *C. felis* (5.1%). Comparing *Cryptosporidium* species distribution between infected cases in immunocompetent and immunocompromised groups showed no significant differences between the two groups. *C. hominis* had the highest frequency in both groups (Table 4). Gastrointestinal symptoms among infected group (n=59) showed regression analysis of the association between *C. hominis* and gastrointestinal manifestations (Table 5), except for a significant association with nausea symptom, (OR) =20.125 (95% CI= 2.133 to 189.879 and P= 0.009).

Table 4. *Cryptosporidium* species distribution among *Cryptosporidium*-positive patients (immunocompetent and immunocompromised).

Symptoms	Patient group*	
	Immunocompetent (n=17) **No. (%)**	**Immunocompromised (n=42)** **No. (%)**
***C.** hominis*	10 (58.8)	21 (50.0)
***C.** parvum*	5 (29.4)	15 (35.7)
C. meleagridis	1 (5.9)	4 (9.5)
C. felis	1 (5.9)	2 (4.8)

*P > 0.05 between the two groups for detected species.

Table 5. Frequency of gastrointestinal manifestations among patients with detected *Cryptosporidium* species (n=59).

Clinical findings	Detected *Cryptosporidium* species			
	C. hominis (n=31)	*C. parvum* (n=20)	*C. meleagridis* (n=5)	*C. felis* (n=3)
Symptoms#				
Nausea	28 (90.3%)*	4 (20%)	1 (20%)	1 (33.3%)
Vomiting	21 (67.7%)	7 (35%)	1 (20%)	1 (33.3%)
Diarrhea	23 (74.2%)	5 (25%)	2 (40%)	1 (33.3%)
Abdominal pain	15 (48.4%)	11 (55%)	3 (60%)	0 (0%)
Weight loss	13 (41.9%)	8 (40%)	3 (60%)	0 (0%)
Signs				
Tender abdomen	15 (48.4%)	9 (45%)	2 (40%)	0 (0%)
Dehydration	8 (25.8%)	8 (40%)	3 (60%)	0 (0%)
Hepatomegaly	6 (19.4%)	5 (25%)	2 (40%)	0 (0%)
Others	11 (35.5%)	6 (30%)	1 (20%)	1 (33.3%)

* P value < 0.05, # Some patients had multiple clinical findings

DISCUSSION

Cryptosporidium has been considered as one of the main pathogens responsible for severe diarrhea and deaths in infants[19,20]. Investigating the cause of moderate-to-severe diarrhea in children under 2 years old, four pathogens were responsible for the majority of cases; rotavirus, *Cryptosporidium*, *Shigella* spp., and heat-stable toxin producing enterotoxigenic *Escherichia coli*[21]. *Cryptosporidium* is transmitted *via* ingestion of oocysts-contaminated food and water or directly through contact with infected person or animal. Following oocysts ingestion, sporozoites are released and infect the host's intestinal epithelial cells, leading to various gastrointestinal manifestations[10].

Cryptosporidiosis generally causes a short-term sickness in immunocompetent persons; however, in immunocompromised persons, the infection could be prolonged due to excystation of thin-shelled oocysts and occurrence of internal autoinfection[22]. The overall infection prevalence in our study was 34%, 46% and 59% as detected by mZN, ELISA and nPCR respectively. According to Omoruyi *et al.*[23], detection percentages of cryptosporidiosis by mZN, ELISA and nPCR diagnostic techniques were 37.1%, 74.3% and 65.7% respectively in HIV-positive patients, while in HIV-negative persons, the detection percentage was 27.2%, 76.8% and 71.2% respectively.

In the present study, prevalence of *Cryptosporidium* infection in studied cases was 59% as detected by nPCR technique, approaching the report by Helmy *et al.*[6] of 49.1% prevalence in Egyptian children but higher than that by El-Badry *et al.*[8] who recorded a prevalence of 19.5%. Both reports involved Egyptian children. In our study prevalence of cryptosporidiosis was significantly higher in immunocompromised children (84%) than immunocompetent ones (34%) and apparently healthy controls (10%), signifying previous suggestions recommending the important role of immunity in controlling this infection[24]. Higher prevalences among immunocompromised patients due to different causes was also recorded in other studies[25,26].

Cryptosporidiosis has great impact on public health due to occupational risks represented by asymptomatic food workers who are considered responsible for asymptomatic carriage[27,28]. Major food-borne cryptosporidial outbreaks among general populations without restriction to immunocompromised patients, were reported in USA and Sweden[29,30].

We detected a significantly higher prevalence in children aged less than 5 years old conforming with other studies; attributing this to an immature intestinal tract mucosal immunity, unhygienic behavior with high exposure and improper hand washing in this age[5,31,32]. In an important observation, Hsu *et al.* remarked that *Cryptosporidium* oocysts expressed high resistance to chlorine disinfection, indicating the importance of boiling potable water in combatting this parasite[33].

Cryptosporidium infection was recorded as relatively high in the age group of 13-36 months[34]. In contrast, the prevalence rates detected in USA, were lowest in younger children and progressively increased in older ages[35]. This was attributed to the greater risk of exposure in elderly persons in developed countries. No significant link to residence was detected in our study suggesting that different methods of transmission as direct contact and water borne infection may be responsible in both rural and urban areas[36].

The principal symptom of cryptosporidiosis is diarrhea, in addition to other gastrointestinal manifestations. In children, especially in developing countries, the course of diarrhea could last for two weeks or more[32]. Among the immunocompetent and immunocompromised children enrolled in our study *Cryptosporidium* oocysts were detected in 63.3% complaining of diarrhoea, confirming the susceptibility of children to this infection. This is substantiated by Abdel Gawad *et al.*[36] who reported a lower rate of infection in an older age group of 37±16.8 years complaining of diarrhea. We recorded a significantly higher occurrence of nausea in *Cryptosporidium*-

infected group (57.6%), consistent with Adler *et al*.[22] who reported a significant association between *Cryptosporidium* infection and nausea.

Cryptosporidium hominis and *C. parvum* were recorded as the major species infecting human population. Other species, like *C. felis*, *C. meleagridis*, *C. muris*, *C. canis* and *C. suis* were reported less frequently[10,11]. We verify the former reports by identifying four of the *Cryptosporidium* species; *C. hominis*, *C. parvum*, *C. meleagridis* and *C. felis*. The prevalent species among both infected immunocompetent and immunocompromised groups was *C. hominis*, suggesting anthroponotic transmission of human cryptosporidiosis in Mansoura City and nearby cities and rural areas. High prevalence of *C. hominis* was also reported in previous studies from Egypt[5,8,37].

According to Sadek[12], about 88.9% of stool samples proved positive in studied children from the Gastroenteritis Unit, Abo El Reesh Pediatric Hospital, Cairo University, were identified as genotype 1 (*C. hominis*). Also from Egypt but from a different locality, El-Hamshary *et al*.[4] showed that most of the detected *Cryptosporidium* species in children from Suez Canal University Hospital, were *C. parvum* (68.4%); while *C. hominis* was detected in only 26.3% of positive cases. Similar prevalence was recorded from immunocompromised inpatients of the oncology, nephrology and pediatrics departments, Suez Canal University hospitals by Soliman and Othman[38]. Using multiplex PCR the authors identified *C. parvum* in 64.7% and *C. hominis* in 29.4% of positive samples. From other countries (Kuwait and Iran) *C. parvum* was the more prevalent species[39,40]. This is understandable because *C. parvum* can infect a range of different species, while *C. hominis* appears to be a particular human parasite with more virulent characteristics than the general *C. parvum*[41]. Other *Cryptosporidium* species with different host ranges also could infect human[42].

Logistic regression analysis of our results revealed that, among the detected *Cryptosporidium* species, *C. hominis* was significantly associated with nausea symptom and may therefore be considered as a risk factor for development of nausea in children. Gastric involvement is thought to occur subsequent to small intestine colonization. It was suggested that conditions associated with hypochlorhydria as in HIV/AIDS, predisposes to gastric cryptosporidial colonization[43]. However, gastric cryptosporidiosis was also reported in immunocompetent patients[44,45]. A small infectious dose of 10-30 oocysts was implicated in the possibility of infection among immunocompetent individuals[46]. Cryptosporidiosis was suggested as a potential cause of unexplained symptoms in infected persons[47], as gastrointestinal symptoms can persist for long periods after the initial infection[48,49].

Our study concludes the high prevalence of *C. hominis* among a sample of children attending Mansoura University Children's Hospital, and associates it mainly with the symptom of nausea. The inclusion of special stains as mZN in routine stool examination is needed for preliminary detection of this emerging parasitic infection.

Author contributions: Abdel Magied SA initiated and designed the study; Elgendy SH collected samples and shared with Elsawey A and Nabih N in performance of laboratory work and writing; Mosaad Y supervised laboratory work.

Conflict of interests: The authors declare that there is no conflict of interests.

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