Journal of the Egyptian Society of Parasitology, Vol.46, No.2, August 2016
J. Egypt. Soc. Parasitol. (JESP), 46(2), 2016: 375 - 386

COPRO-MOLECULAR CHARACTERIZATION OF *CRYPTOSPORIDIUM* SPP. AND GENOTYPES AMONG EGYPTIAN CHILDREN

By

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Abstract

Stool samples from 182 diarrhoeic (symptomatic) children and 100 apparently healthy (asymptomatic) children, matched for age, from Aboul-Reesh Cairo University Pediatrics Hospital were examined by ELISA and by nPCR (targeting COWP gene) for the detection of *Cryptosporidium*. The demographic and environmental data of the diarrhoeic group was recorded. The PCR amplified product of positive cases was then subjected to RFLP by digesting it with the restriction enzyme *Rsa*I. The obtained fragments were resolved by electrophoresis and the bands were visualized and characterized versus a standard. ELISA results demonstrated a prevalence rate of 13.2% (24/182) among diarrhoeic group, and 8% (8/100) among non-diarrheic group, with overall detection rate of 11.3% (32/282). Higher rates of detection were obtained by nested PCR assay among diarrhoeic group 25.8% (47/182) and 16% (16/100) among non-diarrhoeic group with overall detection rate of 22.3% (63/282). Considering nPCR as the reference method, ELISA had a sensitivity of 47.6% and a specificity of 99.1%. *Rsa*I digestion of nPCR product of COWP gene revealed the presence of 2 genotypes: genotype 1 with 4 bands (34, 106, 125 and 285) and genotype 2 in which 3 bands (34, 106 and 401). Among the 63 cases with cryptosporidiosis, 53 (88.3%) had genotype 1, and 7 (11.7%) had genotype 2. The higher prevalence of genotype 1 suggests a relatively greater risk of human source of infection than zoonosis.

Key words: Cryptosporidium, ELISA, nested PCR.

Introduction

Cryptosporidium is a single-celled organism from the Protozoa kingdom. It has gained increasing attention over the last two decades due to its impact on both animal and human health. No therapy has proven to be specifically effective (Chalmers and Davies, 2010). It can cause mild to severe diarrhea. Immuno-competent individuals usually recover within a week, but immunocompromised individuals may be unable to clear the parasites and, consequently, they suffer chronic and debilitating illness (Hijjawi *et al*, 2002; Chalmers and Davies, 2010).

Cryptosporidium has been recognized as an important pathogen in contaminated drinking water as it is resistant to conventional drinking water treatment. The occurrence of cryptosporidiosis outbreaks has been associated with the consumption of contaminated water. This was the case in the largest waterborne disease outbreak which occurred in Milwaukee in 1993 with an estimated 400,000 people reported ill (Hijjawi *et al*, 2002). The taxonomy of *Cryptosporidium* is poorly defined, thus, the understanding of its transmission dynamics is limited. Molecular techniques have revealed extensive genetic variation within *Cryptosporidium* species that led to a better understanding of the taxonomy and zoonotic potential of these variants, as well as the epidemiology of the disease (Xiao and Fayer, 2008).

The present study was designed for immune-molecular identification of *Cryptosporidium* and its genotypes among a group of Egyptian children, and for the validation of diagnostic tests used. Besides, served to

analyze the data variables in order to estimate risks for *Cryptosporidium* detection.

Subjects, Materials and Methods

The present cross sectional study was performed to detect prevailing *Cryptosporidium* copro-antigen and *Cryptosporidium* copro-DNA; to genotype using RFLP-PCR among stool samples from a group of Egyptian children; and to evaluate the two diagnostic tests. A total of 282 children (182 suffering from diarrhea and 100 not suffering from diarrhea) were selected from Aboul-Reesh Cairo University Pediatrics Hospital. The study was ethically approved by ethical committee of Faculty of Medicine, Cairo University and informed consent was obtained from patients or their relatives and parents of young children after they responded to a questionnaire.

A single fecal sample was collected from each child, in addition to demographic, clinical and environmental data. Gross appearance of the collected samples was recorded (WHO, 1991). All fecal samples (diarrhoeic and non-diarrhoeic) were preserved at -20°C with no preservatives added for immunoassays and molecular studies. Part of each fecal sample was subjected to *Cryptosporidium* copro-antigen detection by copro-ELISA using Ridascreen® *Cryptosporidium* ELISA Kit (R-Biopharm AG, Landwehrstr. 54, D-64293 Darmstadt, Germany) according to manufacturer`s instructions.

Fresh frozen fecal samples were processed for genomic DNA extraction after thermal shock (5 cycles of deep freezing in liquid nitrogen & immediately transferred into water bath 95°C each for 5 min.) using Favor Prep-stool DNA isolation Mini Kit (Favorgen Biotech corporation ping-Tung 908, Taiwan, Cat. No. FASTI001). Extracted DNA was amplified targeting *Cryptosporidium* oocyst wall protein (COWP) gene using nested polymerase chain reaction (nPCR), using two sets of primers (table 1). The reaction was done in a total volume of 25µl (Spano *et al,* 1997). The amplified products were visualized with 1.5% agarose gel electrophoresis after ethidium bromide staining. PCR products were digested by *Rsa*I (Fermentas UAB, V. Graiciuno 8, LT-02241 Vilnius, Lithuania) to determine *Cryptosporidium* genotypes. Digested N-COWP fragments were resolved by electrophoresis in 3.2% typing-grade agarose gels containing ethidium bromide, fragments were visualized by UV light.

Statistical analysis: Data were analyzed using the SPSS computer software, version 17.0 (Chicago, IL, USA). Quantitative data was presented as mean ± SD (standard deviation). Difference among groups were compared by student's T test and/or ANOVA followed by Post Hoc test for normally distributed data. Qualitative data was expressed as frequency and percentage. Association between qualitative data was done by using Chi Square test. Risk estimate was done by odds ratio (O.R). Sensitivity, specificity, PVP, NPV & accuracy were calculated with the following formula to analyze: sensitivity: 100 [a/(a+c)]; Specificity: 100x[d/(b+d)]; PVP: 100 x [a/(a+b)]; NPV: 100 x [d/(c+d)], & accuracy: 100 X [a+d/ (a+b+c+d)], where a=true positive samples, b=false positive samples, c=false negative samples & d=true negative samples. For measuring agreement between the methods, Kappa test was done. P value ≤ 0.05 was statistically significant.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the National Research Committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Conflict of Interest: The authors declare that they have neither competing interests nor got any financial support.

Results

Children ages ranged from 4 to 12 years, with a mean of 6.6 years for diarrhoeic children and 7.2 years for non-diarrhoeic ones. ELISA detected *Cryptosporidium* copro-antigen in 24 (13.2%) samples of diarrheic group and in 8 (8%) samples of the non-

diarrheic group with overall detection rate of 11.3 % (32/282). nPCR targeting COWP gene was able to detect *Cryptosporidium* copro- DNA in 47 (25.8%) samples of the diarrheic group and in 16 (16%) samples of non-diarrheic group with overall detection rate of 22.3% (63/282). Among the 47 PCR positive cases of the diarrheic group, 24 of them were negative by ELISA. One case was negative by PCR and positive by ELISA. Among the 16 nPCR positive cases of the non-diarrheic group, 9 were negative by ELISA. One case was negative by PCR and positive by ELISA.

Considering nPCR as a reference method, the performance characteristics of ELISA showed a sensitivity of 47.6%, 99.1% specificity, 93.8% PPV, 86.8 % NPV and over all accuracy of 87.5%. Kappa test of agreement between ELISA and nPCR showed moderate agreement between them.

Using restriction enzyme *Rsa* I, digestion of nPCR product targeting COWP gene (nPCR-RFLP) showed the presence of 2 genotypes: 53 (84.1%) had genotype 1, 7 (11.1%) had genotype 2, and 3 cases could not be digested by *Rsa* I restriction enzyme.

Diarrheic group included 182 children, their sex, stool consistency, the type of water they drink, and history of animal contact was given. The mean age among nPCR positive cases in diarrheic group was 6.4±2.1. According to nPCR results, 47/182 diarrhoeic stool samples were positive for *Cryptosporidium* among them 29 males and 18 females. 21 children were preschool age and 26 were 5 years old and older. Of them 38 were drinking tap water, 4 mineral water & 5 filtered-water. Twenty-five had a history of contact with animals, while 22 did not. Concerning stool consistency, most of nPCR positive samples were watery (23), followed by soft (13) then loose (11).

None of the variables showed significant association with detection of *Cryptosporidium* and its genotypes in stool of diarrheic group. Details were in tables (1 to 9) and figures (1 & 2).

Table 1: PCR target gene and there sequences.

Primers		Sequence	Expected product size (bp)
1ry PCR	BCOWPF	5'ACCGCTTCTCAACAACCATCTTGTCCTC-3'	769-bp
	BCOWR	5'-CGCACCTGTTCCCACTCAATGTAAACCC-3'	
nPCR	Cry-15	5'-GTAGATAATGGAAGAGATTGTG-3'	553-bp
	Cry-9	5'-GGACTGAAATACAGGCATTATCTTG-3'	

Table 2: Mean age of studied groups.

	Diarrheic group (n=182)	Non-diarrheic group (n=100)
Age (Years) mean ± SD	6.6±2.17	7.2±2.20

Data presented as n.

Table 3: Diagnostic yield of nPCR- RFLP of COWP gene and ELISA for detection of *Cryptosporidium* among groups.

			nPCR-RFLP					
			Positive				Negative	Total
			Genotype 1	Genotype 2	Non-typed	Total		
ELISA	Positive	Diarrheic	19	4	0	23	1	24
		Non-Diarrheic	7	0	0	7	1	8
		Total	26 (9.2)	4 (1.4)	0 (0.0)	30 (10.6)	2 (0.7)	32 (11.3)
	Negative	Diarrheic	19	3	2	24	134	158
		Non-Diarrheic	8	0	1	9	83	92
		Total	27 (9.6)	3 (1.05)	3 (1.05)	33 (11.7)	217 (77.0)	250 (88.7)
	Total		53 (18.8)	7 (2.45)	3 (1.05)	63 (22.3)	219 (77.7)	282 (100)

Data presented as n. and (%)

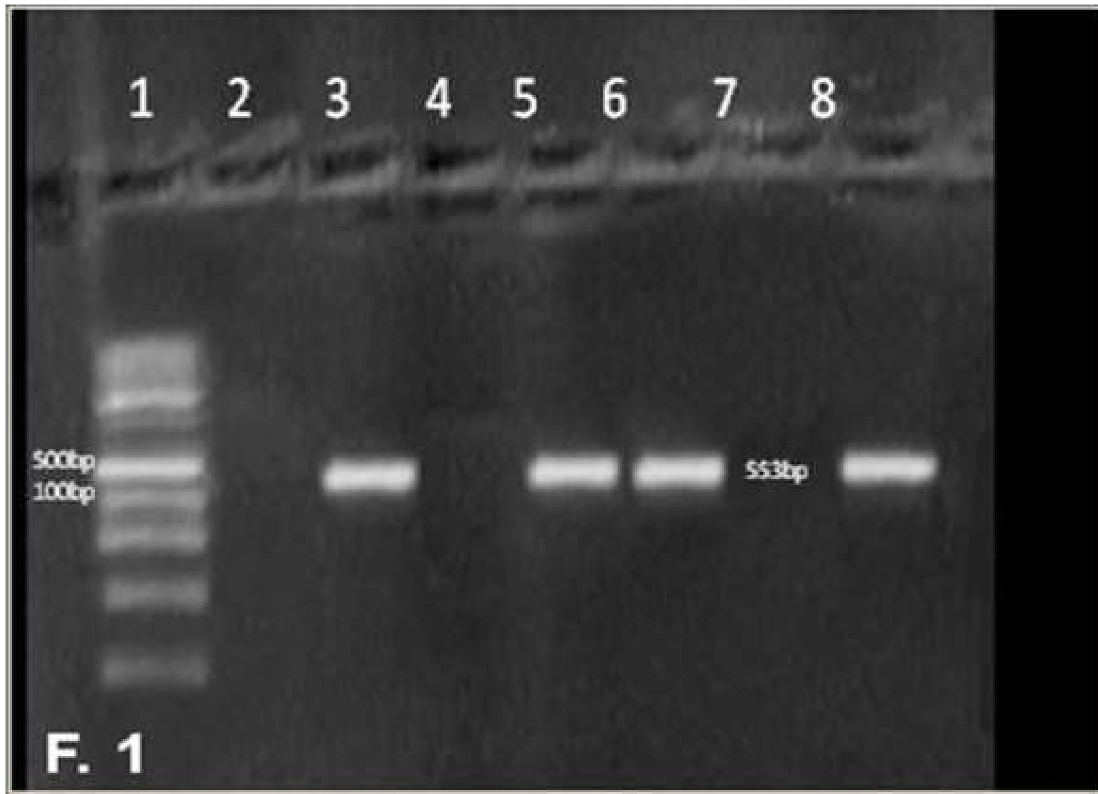

Fig. 1: Agarose gel electrophoresis for products of nPCR targeting COWP gene of *Cryptosporidium* at 553bp. Lane 1: 100 bp DNA molecular weight marker, Lane 2: Negative control, Lane3: Positive control, Lanes 5, 6 & 8 Positive samples, Lanes: 4 & 7 Negative samples.

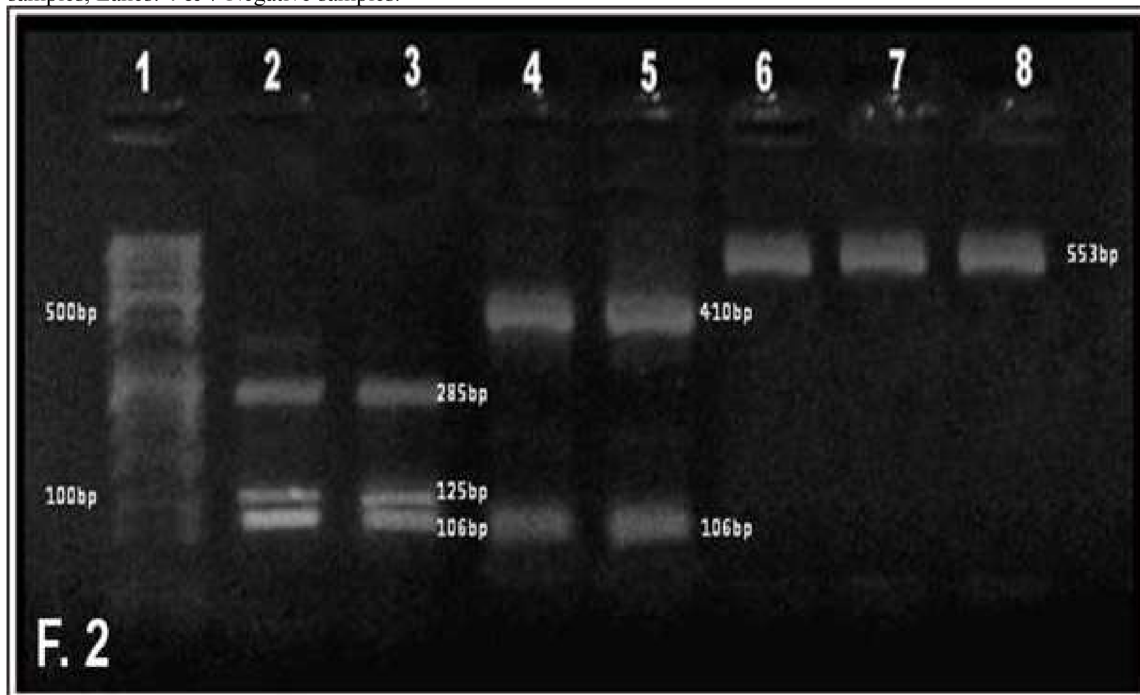

Fig. 2: An agarose gel electrophoresis showing RFLP products after digestion with *Rsa*I endonuclease. Lane 1: 50pb DNA marker ladder, Lane 2 & 3: *C. parvum* genotype 1 digestion products at 34, 106, 125 and 285bp, Lane 4 & 5: *C. parvum* genotype 2 digestion products at 34, 106 and 410 bp (34 band is very small faint and difficult to see), lane 6-8: undigest product of COW product at 553 bp.

Table 4: Diagnostic performance and agreement test between ELISA & nPCR as a reference method.

ELISA	ELISA
Sensitivity	47.6%
Specificity	99.10 %
PPV	93.8 %
NPV	86.8 %
Accuracy	87.5%
Kappa (κ)*	0.566

Table 5: Sex distribution, source of drinking water, history of animal contact and stool consistency among diarrheic group.

Variants		Diarrhoeic group (n=182)
Sex	Males	92 (50.5%)
	Females	90 (49.5%)
Age Group	Up to 5	67 (36.8%)
	>5-12	115 (63.2%)
Type of water	Tap	160 (88%)
	Mineral	8 (4.4%)
	Filter	9 (4.9%)
	Well	5 (2.7%)
Animal contact	Yes	82 (45.1%)
	No	100 (54.9%)
Stool consistency	Watery	92 (50%)
	Soft	37 (20%)
	Loose	53 (30%)

Table 6: Frequency of nPCR results among diarrheic group.

Variants		nPCR Results			*P* value
		+ve (n.)	-ve (n.)	Total (n.)	
Sex	Males	29	63	92	0.09
	Females	18	72	90	
Age group	Up to 5	21	46	67	0.22
	>5-12	26	89	115	
Type of water	Tap	38	122	160	0.03
	Mineral	4	4	8	
	Filter	5	4	9	
	Well	0	5	5	
Animal contact	Yes	25	57	82	0.23
	No	22	78	100	
Stool consistency	Loose	11	42	53	0.29
	Watery	23	69	92	
	Soft	13	24	37	
Total		47	135	182	

Table 7: Data analysis of nPCR positive cases as estimated risk among diarrhoeic group.

Variants		Frequency		OR (95% CI)	*P* value
		+ve (n.)	%		
Sex	Males	29	31.5	1.8 (0.9-3.6)	0.09
	Females	18	20.0		
Age group	Up to 5	21	31.3	1.6 (0.8-3.1)	0.22
	>5-12	26	22.6		
Type of water	Tap	38	23.8	0.3 (0.1-1.3)	0.11
	Mineral	4	50.0		
	Filter	5	55.6	0.8 (0.1-5.4)	1.00
	Mineral	4	50.0		
Animal contact	Yes	25	30.5	1.6 (0.7-3.0)	0.19
	No	22	22.0		
Stool consistency	Watery	23	25.0	0.8 (0.3-1.8)	0.69
	Loose	11	25.8		
	Soft	13	35.1	0.5 (0.2-1.2)	0.15
	Loose	11	25.8		

Table 8: Data analysis of COWP genotypes detected in diarrhoeic group.

Variants		PCR-RFLP Results			*P* value
		Genotype 1 n (%) (n=38)	Genotype 2 n. (%) (n=7)	Non-Typed n (%) (n=2)	
Sex	Males	21(72.4)	6(20.7)	2(6.9)	0.16
	Females	17(94.4)	1(5.6)	0(0.0)	
Age group	Up to 5	18(85.7)	2(9.5)	1(4.8)	0.65
	>5-12	20(76.9)	5(19.2)	1(3.8)	
Type of water	Tap	30(78.9)	6(15.8)	2(5.3)	0.85
	Mineral	4(100.0)	0(0.0)	0(0.0)	
	Filter	4(80.0)	1(20.0)	0(0.0)	
	Well	0(0.0)	0(0.0)	0(0.0)	
Animal contact	Yes	21(84.0)	3(13.0)	1(4.0)	0.83
	No	17(77.3)	4(18.2)	1(4.5)	
Stool consistency	Loose	10(90.9)	1(9.1)	0(0.0)	0.64
	Watery	17(73.9)	5(21.7)	1(4.3)	
	Soft	11(84.6)	1(7.7)	1(7.7)	
Total		38(80.9)	7(14.9)	2(4.3)	

Data presented as n. and (%)

Table 9: Data analysis for COWP genotypes as estimated risks among diarrhoeic group.

Variants		Frequency		OR (95% CI)	*P* value
		Genotype 1 n. (%)	Genotype 2 n. (%)		
Sex	Males	21(77.8)	6(22.2)	0.2(0.02-1.9)	0.22
	Females	17(94.4)	1(5.6)		
Age group	Up to 5	18(90.0)	2(10.0)	2.3(0.4-13.1)	0.44
	>5-12	20(80.0)	5(20.0)		
Type of water	Tap	30(83.3)	6(16.7)	0.8(0.7-1.0)	1.00
	Mineral	4(100.0)	0(0.0)		
	Filter	4(80.0)	1(20.0)	1.3(0.8-1.9)	1.00
	Mineral	4(100.0)	0(0.0)		
Animal contact	Yes	21(87.5)	3(12.5)	1.6(0.3-8.4)	0.69
	No	17(81.0)	4(19.0)		
Stool consistency	Watery	17(77.3)	5(22.7)	2.9(0.3-28.9)	0.64
	Loose	10(90.9)	1(9.1)		
	Soft	11(91.7)	1(8.3)	0.9(0.1-16.5)	1.00
	Loose	10(90.9)	1(9.1)		

Data presented as n. and (%).

Discussion

Cryptosporidiosis was a prevailing enteric pathogen among both diarrhoeic and non-diarrhoeic Egyptian children. Molecular assays improved the ability to detect *Cryptosporidium*. In agreement with the present study El-Hamshary *et al* (2008) found that the prevalence of *Cryptosporidium* was 25% among diarrheic Egyptian children using multiplex PCR. Higher rate of *Cryptosporidium* prevalence (32.4%) by nPCR was reported by Salyer *et al* (2012). Rizk and Soliman (2001), Abdel-Messih *et al.* (2005), El-Mohamady *et al.* (2006) found that 13.9%, 17% & 15% of diarrheic children by ELISA had cryptosporidiosis respectively.

Tahira *et al* (2012) and Al-Hindi *et al* (2007) reported a prevalence of 11.6% and 16.3% in children with diarrhea by using ELISA respectively. The prevalence rate of cryptosporidiosis was 18% in a study among school children and hospital patients in South Africa (Samie *et al*, 2006), while in Kenya, the percentage was 25% (Gatei *et al*, 2006).

In contrast, higher rates were reported by

Garcia *et al.* (2003) reported 21.2% (85/401) immuno-reactive for *Cryptosporidium*. Hel=my *et al* (2004) stated that 44% were positive for this parasite. Sulaiman *et al* (2005) reported 94%. Mirzaei (2007) who revealed that 25.6% of diarrheic children and 3.7% of non-diarrheic ones infected with *C. parvum*. Al-Shibani *et al.* (2009) reported the prevalence of *C. parvum* as 24% and Al-Shamiri *et al.* (2010) reported 34.7%. Youssef *et al* (2000) and Nair *et al* (2008) reported much lower prevalence rates (1.5% & 3%, respectively) than in the present studied children with gastroenteritis.

The discrepancies in the reported prevalence of other studies and the current study`s results may be attributed to differences in population demographics, behavioral, environmental and socioeconomic factors, as well as differences in diagnostic methods, and time of the study (summer vs. winter).

There was no commonly approved standard for the detection of *Cryptosporidium* in stool specimens (Tahira *et al,* 2012). In the present study, the nPCR was considered the reference method. Sensitivity, specificity & diagnostic accuracy of ELISA were 47.6%, 99% and 87.5% respectively in comparison to nPCR. The difference in sensitivity of immunoassays may be because the available copro-antigen detection ELISA kits recognized different sets of surface epitopes by the use of mAbs which in turn may or may not react with antigens of different *Cryptosporidium* species. In addition, fecal immunoassays detection limit was reported to be 3×10^{5}-10^{6} (Smith, 2008).

The current data indicated that the nPCR method succeeded in detecting a larger number of cases than ELISA method among both the diarrheic group (47 vs. 24) and the non-diarrheic group (16 vs. 8). Kappa test revealed that there was moderate agreement between the ELISA and nPCR.

In Egypt, El-Shazly *et al.* (2002); Abdel-Baki *et al.* (2004) and El-Hamshary *et al.* (2008) found that ELISA showed lower sensitivity and accuracy in relation to PCR. The first study found that the sensitivity, specificity and accuracy of ELISA detection of *Cryptosporidium* in relation to detection of DNA in stool by PCR were 84.2%, 96% and 88.8%, for the first study. Second study revealed 85.7% sensitivity, 100% specificity, 100% diagnostic accuracy, 100% PPV and 98.9% NPV. The third one found copro-antigen ELISA was more sensitive (85.7%) and specific (100%) with 96.5% accuracy. Besides, copro-antigen detection with ELISA was more sensitive (85.7%) and specific (100%) with 96.5% accuracy in detection of *Cryptosporidium* compared with PCR (El-Settawy and Fathy, 2012). The fact that mixed infections, re-infections, & cross-reaction with intestinal and gastric *Cryptosporidium* species may occur in human cryptosporidiosis (Cama *et al*, 2008) could be an explanation of why a higher sensitivity using molecular detection methods was obtained.

Socio-behavioral and environmental factors possibly play a role in risk exposure and /or affect the prevalence of *Cryptosporidium* infection. These factors may affect susceptibility, but not the pathogenicity or course of the disease. The mechanisms of these factors in cryptosporoidiosis in children were not yet clear (Roy *et al*, 2004; Yoder and Beach, 2010).

In the present study, there was no significant association between *Cryptosporidium* and its genotypes and the following variables: sex, age, clinical picture, type of drinking water, contact with animals and stool consistency. Some studies did not find an animal association, confirming the importance of other modes of transmission and perhaps the presence of different *Cryptosporidium* species (Mikhail *et al*, 1989; Youssef *et al,* 2008). Yu *et al.* (2004) observed a statistically significant association between farmers and their animals infected with *C. parvum*, proving that the contact with domestic animals acted as a risk factor in zoonotic infection. Hassl *et al.* (2001), Park *et al* (2006) and Mirzaei (2007) reported that the contamination of drinking water was an im-

portant risk factor in the prevalence of *Cryptosporidium* spp.

The highest rate of prevalence of *Cryptosporidium* was observed in age 5-12 years old with no statistical significance. These results were in agreed with studies conducted by Abou-El-Magd *et al.* (1986); Abdel-Messih *et al.* (2005); Al-Shamiri *et al.* (2010); Iqbal *et al.* (2011); In contrast, Casemore *et al* (1990) and El-Helalya *et al* (2012) found that the peak incidence of cryptosporidiosis was in children aged 1-5 years. Thus, children 6 to 12 years old may be more exposed to infection by *Cryptosporidium*, but the reason is unknown, this may be because they lack knowledge about hygiene, eat without washing their hands, play in soil and sewage water. This may be a reflection of their immature immune system (lacking immunity due to few prior exposures), immaturity of gut mucosa or due to the fact that diarrhoeic children attend a physician more frequently, thus increasing probability of the *Cryptosporidium* detection (El-Helalya *et al*, 2012). The mean age of children infected with genotype 1 was comparable to that of those infected with genotype 2, suggesting nearly similar age susceptibility. In contrast, Bushen *et al* (2007) reported that *C. hominis* was found at a somewhat earlier age than *C. parvum*.

Regarding sexes, the present study showed that boys and girls had similar positive rates without significant association, which was concurrent with Abdel-Messih *et al* (2005) and El-Helalya *et al* (2012). Samie *et al* (2006) in immuno-competent children found that *C. hominis* was equally distributed between males and females, whereas *C. parvum* was commonest among females (75%) than males (25%). In contrast, Park *et al* (2006) reported that the infection was more common among males than females, which could be due to a larger sample size of males than females, or may be due to more frequent playing of male children in gardens and farms with soil and animals.

In the present study, *Cryptosporidium* was detected in all types of stool, and the obtained results indicate that *Cryptosporidium* was more prevalent in watery stool samples followed by soft stool samples; however, there was no statistical significant association between *Cryptosporidium* infection and stool consistency.

Interestingly, in the present study, cryptosporidiosis was detected in 16/100 non-diarrheic (formed stool) samples. Several authors reported a correlation between watery diarrhea, cholera-like illness and the presence of *Cryptosporidium*. Chauret *et al* (1999) and Chalmers and Davies (2010). However, Youssef *et al* (2008) and El-Helalya *et al* (2012) reported that soft stool was the commonest form of *Cryptosporidium* diarrheic stool. There is a possibility of occurrence of asymptomatic carrier state for *Cryptosporidium* in non-diarrhoeic (formed stool) immuno-competent and immuno-compromised patients (Janoff *et al,* 1990; Chappell *et al,* 1996; Albraiken *et al*, 2003).

Chalmers and Davies (2010) reported that asymptomatic cryptosporidiosis could cause retardation in weight gain and adversely affect growth. The asymptomatic cryptosporidiosis could cause mucosal damage, altering the intestinal function enough to cause nutrient malabsorption. Unfortunately, malnourished children without diarrhea were seldom tested for the presence of enteric pathogens.

Molecular tools, mainly PCR-based, were used to identify different types of *Cryptosporidium* targeting the amplification of a gene which gives information about the species or genotype of the isolate. These molecular tools have been helpful in enhancing our knowledge and for the understanding of the taxonomy, host range and transmission routes of *Cryptosporidium* and the epidemiology of human disease. Also, *Cryptosporidium* genotyping was used to understand the different environmental routes of transmission, leading toward improved strategies for

prevention and surveillance of cryptosporidiosis (Jex *et al*, 2008).

In the present study genotype 1 was found more frequently in all cryptosporidiosis patient groups, infecting 88.3%, while genotype 2 was 11.7%. None showed mixed infection. As genotype 1 is the human genotype and genotype 2 infected different animals as well (Pedraza-Diaz *et al,* 2000) the present findings indicate that human source of infection is more common than zoonotic sources in the studied groups. This predominance in infections caused by genotype 1 was in accordance with Xiao *et al* (2001). Similar data were reported form Europa as the Netherlands (Homan *et al*, 1999), France (Guyot *et al,* 2001), Northern Ireland (Lowery *et al,* 2001), Denmark (Enemark *et al,* 2002), Switzerland (Fretz *et al*, 2003; Glaeser *et al*, 2004), the Czech Republic (Hajdusek *et al*, 2004), and Scotland (Mallon *et al,* 2003a) albeit that other studies were on a much smaller scale and included a maximum of 135 patients (Mallon *et al*, 2003b).

In Arab countries, apart from zoonotic cryptosporidiosis in Egypt (Youssef *et al*, 2008; Helmy *et al*, 2015; Shalaby and Shalaby, 2015), its prevalence was reported in Saudi Arabia (Hawash *et al*, 2014), Kuwait (Majeed and Alazemi, 2014) and Lebanon (Osman *et al*, 2015).

In contrast, two studies of sporadic cases and small outbreaks in the United Kingdom showed that genotype 2 isolates were predominant (McLauchlin *et al,* 1999; 2000; Guyot *et al*, 2001). Nichols *et al.* (2006) found that 50.3% were *C. hominis* infected cases and 45.6% with *C. parvum*. Besides, *C. hominis* was more prevalent than *C. parvum* in USA, Canada, Australia, Japan and developing countries where molecular tools were used to identify specimens (Xiao and Fayer, 2008). In contrast to the present results found that apart from *C. parvum* and *C. hominis* were reported in immune-competent patients (Xiao *et al,* 2001) and patients with HIV (Guyot *et al,* 200; Cama *et al,* 2003; Xiao *et al,* 2004).

In the present study, three positive samples by PCR could not be cut by restriction enzyme, that may be due single base mismatch (T to C substitution) which occurred in the Cry9 COWP mutation in the genome and this need sequencing (Spano *et al,* 1997; Pedraza-Diaz *et al*, 2000). The finding here of a heterogeneous group of *Cryptosporidium* species in humans without immunocompromising illness suggested that unusual species may play a role in human infections.

In the present study, all cases of genotype 2 were found among diarrheic group, which agreed with results of Xiao *et al* (2001) who explained that genotype 2 was more virulent than genotype 1. In contrast, Bushen *et al* (2007) reported that children were no more likely to be symptomatic when infected with genotype 2.

Based on the obtained results we can conclude that *Cryptosporidium* is an important cause of diarrhea among Egyptian children with a prevalence of 22.3%. Asymptomatic *cryptosporidium* infection was detected among non-diarrhoeic children with a prevalence of 16%. These findings can be attributed to a higher sensitivity of molecular detection of copro-DNA using nPCR over immuno-detection of copro-antigen by ELISA among both diarrhoeic and non-diarrheic fecal samples.

Conclusion

The COWP nPCR and RFLP assay proved useful for diagnosis and genotyping of *Cryptosporidium*. Both genotype 1 and genotype 2 are present in Egyptian children. Genotype 1 was found to be relatively more prevalent than genotype 2 among all groups of patients examined, suggesting a relatively greater risk of human source of infection than zoonosis. While, genotype 2 was detected only in diarrhoeic samples which might explain that the genotype 2 was more virulent than genotype 1.

Recommendation

No doubt, more genetic characterization of *Cryptosporidium* with representative samples from all Egyptian communities and lo-

calities is highly recommended to determine the molecular epidemiology of *Cryptosporidium* genotypes and to assess cross-species transmission, and consequently, one can suggest feasible control measures for this public health problem particularly among the children.

References

Abdel Messih, IA, Wierzba, TF, Abu-Elyazeed, R, Ibrahim, AF, Ahmed, SF, *et al*, 2005: Diarrhea associated with *Cryptosporidium parvum* among children of the Nile River Delta in Egypt. J. Trop. Pediat., 51, 3:154-9.

Abdel-Baki, M, Younis, T, Habib, K, Ramadan, NI, Metwally, DM, *et al*, 2004: Molecular identification of coproantigen of *Cryptosporidium parvum* compared to conventional staining techniques. J. Egypt Soc. Parasitol. 34, 3:967-78.

Abou-El-Magd, LA, Abou Shady, OA, 1986: Preliminary study of human cryptosporidiosis. J. Egypt Soc. Parasitol. 16:573-7.

Al Braiken, FA, Amin, A, Beeching, NJ, Hommel, M, Hart, CA, 2003: Detection of *Cryptosporidium* amongst diarrhoeic and asymptomatic children in Jeddah, Saudi Arabia. Ann. Trop. Med. Parasitol. 97, 5:505-10.

Al Hindi, AI, El Manama, AA, Elnabris, KJ A, 2007: Cryptosporidiosis among children attending Al-Nasser Pediatric Hospital, Gaza, Palestine. Turk. J. Med. Sci., 37, 6:367-72.

Al-Shamiri, AH, Al-Zubairy, AH, Al-Mamari, RF, 2010: The Prevalence of *Cryptosporidium* spp. in Children, Taiz District, Yemen. Iranian J. Parasitol. 5, 2:26-32.

Al-Shibani, LA, Azazy, AA, El-Taweel, HA, 2009: *Cryptosporidium* spp. and cryptosporidiosis. Microbiol. Rev. 50:458-83.

Bushen, OY, Kohli, A, Pinkerton, RC, Dupnik, K, Newman, RD, *et al*, 2007: Heavy cryptosporidial infections in children in northeast Brazil: comparison of *Cryptosporidium hominis* and *Cryptosporidium parvum*. Trans. R. Soc. Trop. Med. Hyg. 101, 4:378-84.

Cama, VA, Bern, C, Sulaiman, IM, Gilman, R H, Ticona, E, *et al*, 2003: Cryptosporidium species and genotypes in HIV-positive patients in Lima, Peru. J. Eukaryot. Microbiol. 50:531-3.

Cama, VA, Bern, C, Roberts, J, Cabrera, L, Sterling, CR, *et al*, 2008: *Cryptosporidium* species and subtypes and clinical manifestations in children, Peru. Emerg. Infect. Dis. 14, 10:1567-74.

Casemore, DP, 1990: Laboratory methods for diagnosing cryptosporidiosis. J. Clin. Pathol. 44: 445-51.

Chalmers, RM, Davies, AP, 2010: Minireview: Clinical cryptosporidiosis. Exp. Parasitol. 124: 138-46.

Chappell, CL, Okhuysen, PC, Kettner, C, Sterling, CR, 1996: *Cryptosporidium parvum* metalloaminopeptidase inhibitors prevent in vitro excystation. Antimicrob agents Chemother. 40, 12: 2781-4.

Chauret, C, Springthorpe, S, Sattar, S, 1999: Fate of *Cryptosporidium* oocysts, Giardia cysts, and microbial indicators during wastewater treatment and anaerobic sludge digestion. Can. J. Microbiol. 45, 3:257-62.

El Mohammady, H, Abdel Messih, IA, Youssf, FG, Said, M, Farag, H, *et al*, 2006: Enteric patho-gens associated with diarrhea in children in Fayoum, Egypt. Diag. Microbiol. Infect. Dis. 56:1-5.

El-Hamshary, EM, El-Sayed, HF, Hussein, E M, Soliman, RS, 2008: Comparison of Polymerase Chain Reaction, immunochromatographic assay and staining techniques in diagnosis of cryptosporidiosis. Parasitologists United Journal (PUJ). 1, 2:77-86.

El-Helalya, N, Alyb, M, Attia, S, 2012: Detection of *Cryptosporidium* infection among children with diarrhea. NY Sci. J. 5, 7:68-76.

El-Settawy, MA, Fathy, GM, 2012: Evaluation and comparison of PCR, Coproantigen ELISA and microscopy for diagnosis of *Cryptosporidium* in human diarrhoeic specimens. J. Am. Sci. 8, 12:2505-11.

El-Shazly, AM, Gabor, A, Mahmoud, MSE, Abdel Aziz, SS, Saleh, WA, 2002: The use of Zhiel-Neelsen stain, Enzyme-linked immunosorbent assay and nested polymerase chain reaction in diagnosis of cryptosporidiosis in immuno-competent, compromised patients. J. Egypt. Soc. Parasitol. 32, 1:155-66.

Enemark, HL, Ahrens, P, Juel, CD, Petersen, E, Petersen, RF, *et al*, 2002: Molecular characteri-zation of Danish *Cryptosporidium parvum* isolates. Parasitol. 125:331-41.

Fretz, R, Svoboda, P, Ryan, UM, Thompson, RC, Tanners, M, *et al*, 2003: Genotyping of *Cryptosporidium* spp. isolated from human stool samples in Switzerland. Epidemiol Infect. 131: 663-7.

Garcia, LS, Shimizu, RY, Novak, S, Carroll, M, Chan, F, 2003: Commercial assay for detec-

tion of *Giardia lamblia* and *Cryptosporidium parvum* antigens in human fecal specimens by rapid solid-phase qualitative immunochromatography. J. Clin. Microbiol. 41, 1:209-12.

Gatei, W, Wamae, CN, Mbae, C, Waruru, A, Mulinge, E, *et al*, 2006: Cryptosporidiosis: prevalence, genotype analysis, and symptoms associated with infections in children in Kenya. Am. J. Trop. Med. Hyg. 75, 1:78-82.

Glaeser, C, Grimm, F, Mathis, A, Weber, R, Nadal, D, *et al,* 2004: Detection and molecular characterization of *Cryptosporidium* spp. isolated from diarrheic children in Switzerland. Pediatr. Infect Dis. J. 23:359-61.

Guyot, K, Follet-Dumoulin, A, Lelievre, E, Sarfati, C, Rabodonirina, M, *et al*, 2001: Molecular characterization of *Cryptosporidium* isolates obtained from humans in France. J. Clin. Microbiol. 39:3472-80.

Hajdusek, O, Ditrich, O, Slapeta, J, 2004: Molecular identification of *Cryptosporidium* spp. in animal and human hosts from the Czech Republic. Vet. Parasitol. 122:183-92.

Hassl, A, Benyr, G, Sommer, R, 2001: Occurrence of *Cryptosporidium* sp. oocysts in fecal & water samples in Austria. Acta Trop. 80:145-9.

Hawash, Y, Dorgham, LSh, Al-Hazmi, AS, Al-Ghamdi, MS, 2014: Prevalence of Cryptosporidium-associated diarrhea in a high altitude-community of Saudi Arabia detected by conventional and molecular methods. Korean J Parasitol. 52, 5:479-85.

Helmy, MM, Rashed, LA, El-Garhy, MF, 2004: Molecular characterization of *Cryptosporidium parvum* isolates obtained from humans. J. Egypt. Soc. Parasitol. 34, 2:447-58.

Helmy, YA, VON Samson-Himmelstjerna, G, Nöckler, K, Zessin, KH, 2015: Frequencies and spatial distributions of *Cryptosporidium* in livestock animals and children in the Ismailia Province of Egypt. Epidemiol. Infect. 143, 6:1208-18.

Hijjawi, NS, Meloni, BP, Ryan, UM, Olson, M E, Thompson, RC, 2002: Successful in vitro cultivation of *Cryptosporidium andersoni*: Evidence for the existence of novel extracellular stages in the life cycle and implications for the classification of *Cryptosporidium*. Int. J. Parasitol. 32:1719-26.

Homan, W, van Gorkom, T, Kan, YY, Hepener, J, 1999: Characterization of *Cryptosporidium parvum* in human and animal feces by single-tube nested polymerase chain reaction and restriction analysis. Parasitol. Res. 85:707-12.

Iqbal, J, Khalid, N, Hira, PR, 2011: Cryptosporidiosis in Kuwaiti children: association of clinical characteristics with *Cryptosporidium* species and subtypes. J. Med. Microbiol. 60:647-52.

Janoff, EN, Mead, PS, Mead, JR, Echeverria, P, Bodhidatta, L, *et al*, 1990: Endemic *Cryptosporidium* and *Giardia lamblia* infections in a Thai orphanage. Am. J. Trop. Med. Hyg. 43, 3: 248-56.

Jex, AR, Smith, HV, Monis, PT, Campbell, B E, Gasser, RB, 2008: *Cryptosporidium*-biotechnological advances in the detection, diagnosis and analysis of genetic variation. Biotechnol. Adv. 26:304-17.

Lowery, CJ, Millar, BC, Moore, JE, Xu, J, Xiao, L, *et al*, 2001: Molecular genotyping of human cryptosporidiosis in Northern Ireland: epidemiological aspects and review. Ir. J. Med. Sci. 170:246-50.

Majeed, QA, Alazemi, MS, 2014: Preliminary study on cryptosporidiosis in livestock from Kuwait. J. Egypt. Soc. Parasitol. 44, 2:389-92.

Mallon, M, MacLeod, A, Wastling, J, Smith, H, Reilly, B, *et al*, 2003a: Population structures and the role of genetic exchange in the zoonotic pathogen *Cryptosporidium parvum*. J. Mol. Evol. 56:407-17.

Mallon, ME, MacLeod, A, Wastling, JM, Smith, H, Tait, A, 2003b: Multilocus genotyping of *Cryptosporidium parvum* Type 2: population genetics and substructuring. Infect. Genet. Evol. 3:207-18.

Mc-Lauchlin, J, Amar, C, Pedraza-Diaz, S, Nichols, GL, 2000: Molecular epidemiological analysis of *Cryptosporidium* spp. in the United Kingdom: results of genotyping *Cryptosporidium* spp. in 1,705 fecal samples from humans and 105 fecal samples from livestock animals. J. Clin. Microbiol. 38:3984-90.

Mc-Lauchlin, J, Pedraza-Diaz, S, Amar-Hoetzeneder, C, Nichols, GL, 1999: Genetic characterization of *Cryptosporidium* strains from 218 patients with diarrhea diagnosed as having sporadic cryptosporidiosis. J. Clin. Microbiol. 37: 3153-8.

Mikhail, IA, Hyams, KC, Podgore, JK, Haberberger, RL, Boghdadi, AM, *et al*, 1989: Microbiologic and clinical study of acute diarrhea in children in Aswan, Egypt. Scand. J. Infect. Dis. 21:59-65.

Mirzaei M, 2007: Prevalence of *Cryptosporidium* sp. infection in diarrhea and non-diarrheic humans in Iran. Korean J. Parasitol. 45, 2:133-7.

Nair, P, Mohamed, JA, DuPont, HL, Figueroa, JF, Carlin, L, *et al*, 2008: Epidemiology of cryptosporidiosis in North American travelers to Mexico. Am. J. Trop. Med. Hyg. 79, 2:210-14.

Nichols, RA, Campbell, BM, Smith, HV, 2006: Molecular fingerprinting of Parasites in 3 Yemeni orphanages: prevalence, risk and morbidity. J. Egypt. Soc. Parasitol. 39, 1:327-37.

Osman, M, El Safadi, D, Benamrouz, S, Guyot, K, Dei-Cas, E, *et al*, 2015: Initial data on the molecular epidemiology of cryptosporidiosis in Lebanon. PLoS One. May 7;10(5):e0125129. 10. 1371/journal.pone.0125129.

Park, JH, Kim, HJ, Guk, SM, Shin, EH, Kim, JL, *et al*, 2006: survey of cryptosporidiosis among 2,541 residents of 25 coastal islands in Jeollanam-Do (Province), Republic of Korea. Korean J. Parasitol. 44, 4:367-72.

Pedraza-Diaz, S, Amar, C, Mc Lauchlin, J, 2000: The identification and characterisation of an unusual genotype of *Cryptosporidium* from human feces as *Cryptosporidium meleagridis*. FEMS Microbiol. Lett. 189:189-94.

Rizk, H, Soliman, M, 2001: Coccidiosis among malnourished children in Mansoura, Dakahlia Governorate, Egypt. J. Egypt. Soc. Parasitol. 31, 3:877-86.

Roy, SL, DeLong, SM, Stenzel, SA, Shiferaw, B, Roberts, JM, *et al*, 2004: Risk factors for sporadic cryptosporidiosis among immunocompetent persons in the United States from 1999 to 2001. J. Clin. Microbiol. 42: 2944-51.

Salyer, SJ, Gillespie, TR, Rwego, IB, Chapman, CA, Goldberg, TL, 2012: Epidemiology and molecular relationships of *Cryptosporidium* spp. in people, primates, and livestock from Western Uganda. PLoS Negl. Trop. Dis. 6, 4: e1597.

Samie, P, Bessong, O, Obi, CL, Sevilleja, JE, Stroup, S, *et al*, 2006: *Cryptosporidium* species: Preliminary descriptions of the prevalence and genotype distribution among school children and hospital patients in the Venda region, Limpopo Province, South Africa. Exp. Parasitol. 114, 4: 314-22.

Shalaby, NM, Shalaby, NM, 2015: *Cryptosporidium parvum* infection among Egyptian school children. J. Egypt. Soc. Parasitol. 45, 1:125-31.

Smith, S, Shaw, J, Nathwani, D, 2008: Nitazoxanide for cryptosporidial infection in Crohn's disease. Gut 57, 8:1179-80.

Spano, F, Putignani, L, McLauchlin, J, Casemore, DP, Crisanti, A, 1997: PCR-RFLP analysis of the *Cryptosporidium* oocyst wall protein (COWP) gene discriminates between *C. wrairi* and *C. parvum*, and between *C. parvum* isolates of human and animal origin. FEMS Microbiol. Lett. 150:209-17.

Sulaiman, IM, Hira, PR, Zhou, L, Al-Ali, FM, Al-Shelahi, FAH, *et al*, 2005: Unique endemicity of cryptosporidiosis in children in Kuwait. J. Clin. Microbiol. 43, 6:2805-9.

Tahira, F, Khan, HM, Shukla, I, Shujatullah, F, Malik, MA, *et al*, 2012: Prevalence of *Cryptosporidium* in Children with Diarrhea in North Indian Tertiary Care Hospital. J. Comm. Med. Hlth. Edu. 2:136-9.

WHO, 1991: Parasitolology-Laboratory Manuals: Basic Laboratory Methods in Medical Parasitology, Geneva, Switerland.

Xiao, L, Fayer, R, 2008: Molecular characterisation of species and genotypes of *Cryptosporidium* and *Giardia* and assessment of zoonotic transmission. Int. J. Parasitol. 38, 1239-55.

Xiao, L, Bern, C, Limor, J, Sulaiman, I, Roberts, J, *et al*, 2001: Identification of 5 types of *Cryptosporidium* parasites in children in Lima, Peru. J. Infect. Dis. 183:492-7.

Xiao, L, Fayer, R, Ryan, U, Upton, SJ, 2004: *Cryptosporidium* taxonomy: recent advances and implications for public health. Clin. Microbiol. Rev. 17:72-97.

Yoder, JS, Beach, MJ, CDC, 2010: Cryptosporidiosis surveillance: United States, 2006-2008. 59, 6:1-14.

Youssef, FG, Adib, I, Riddle, MS, Schlett, C D, 2008: A review of cryptosporidiosis in Egypt. J. Egypt. Soc. Parasitol. 38, 1:9-28.

Youssef, M, Shurman, A, Bougnoux, M, Rawashdeh, M, Bretagne, S, *et al*, 2000: Bacterial, viral and parasitic enteric pathogens associated with acute diarrhea in hospitalized children from northern Jordan. Immunol. Med. Microbiol. 3: 257-63.

Youssef, FG, Adib, I, Riddle, MS, Schlett, C D, 2008: A review of cryptosporidiosis in Egypt. J. Egypt. Soc. Parasitol. 38, 1:9-28

Yu, JR, Lee, JK, Seo, M, Kim, SI, Sohn, WM, *et al*, 2004: Prevalence of cryptosporidiosis among the villagers and domestic animals in several rural areas of Korea. Korean J. Parasitol. 42:1-6.