

CRYPTOSPORIDIOSIS PROVOKING DIARRHEA AMONG INFANTS, TODDLERS AND PRESCHOOL CHILDREN

By

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Abstract

Cryptosporidium intestinal coccidian is a preminent account for infectious diarrhea with reference to children. Oocyst may escape ordinary microscopic analysis or immune detection; molecular techniques are of convenient use. This study intent to detect cryptosporidiosis infection among children, and debates risks for infection. A total of 189 children with age range from under one to five complaining of gastrointestinal manifestations were examined coproscopically, by acid fast stain, ELISA and nested PCR (nPCR) for *Cryptosporidium* from June to September. Infection was proved by nPCR among 13 (6.8%) samples. ELISA sensitivity merit stool staining (77%, 53.8%, respectively), however all positive samples by microscopy were settled by nPCR. Age groups ($P = 0.02$) were statistically associated with infection. Source of water ($OR=0.069$, 95% $CI= 0.004/1.165$, $P = 0.01$) and presence of undigested food particles on microscopy ($OR= 1.058$, 95% $CI= 0.521/6.154$, $P = 0.02$) were risks for infections. No other variables were predicated risks. Infection was potent among preschool children (46.2%), toddlers (38.5%) and then infants (15.4%). Cryptosporidiosis should be doubtful among children with diarrhea during preschool age. Source of water for ordinary drinking activities or for infants with non-exclusive breast or complementary feeding should be considered as hazards aspects for acquiring *Cryptosporidium*.

Key words: Cryptosporidiosis, Children, Diarrhea, ELISA, Stain, nPCR

Introduction

Cryptosporidium is distributed universally among all ages; however it is an imperative infectious disease common in children (Fathy *et al*, 2014). The two species *parvum* and *hominis* are incorporated to motif of human infections (Bouزيد *et al*, 2013). It is linked to wide range of clinical manifestations depending on the nutrition, immunity state, and age of patients; however diarrhea is a common characteristic of the disease (Mor and Tzipori. 2008; Bouزيد *et al*, 2013).

Infection is a common pediatric barrier; during the early two years it is linked to increase mortality (Bouزيد *et al*, 2018). In addition to variety of childhood impediments; dehydration, under nutrition, weight loss, malabsorption, affecting growth of children by expanded diarrhea (Nath *et al*, 2015; Khalil *et al*, 2018).

The story of malnutrition and cryptosporidiosis is a vicious circle, under nutrition aggravates infection and decrease immunity. On the other hand, cryptosporidiosis affects nutrients absorption causing malnutrition and lengthens the disease duration enforcing reinfections (White *et al*, 2015). Using molecular techniques for confirmation of infection is helpful (Helmy *et al*, 2014; Ghieth *et al*, 2018).

At Beni-Suef community a high frequency of infection was reported in older age groups (Abdel Gawad *et al*, 2018), thus making a spotlight on the symptomatic under-five children was pivotal for better management of cryptosporidiosis induced manifestation.

Subjects and Methods

Ethical circumstances and study population: This cross-sectional study including 189 children with age up to 5 attending the Beni-Suef Pediatrics Outpatients Clinic and complaining of gastro-intestinal com-

plains. A constructed questionnaire was fulfilled by parents of children. All parents were previously notified about the research aim; they found no trouble to ignore the study. Samples collection was done only by their approval.

All children were asked to collect from one to three stool samples. It was divided into three parts, first one for doing microscopy by direct wet mount and concentration technique after adding saline and Lugol's iodine to detect the intestinal protozoans (oo)cysts, ova and any stages of helminthes using x10 & x40 objectives. For microscopic diagnosis of cryptosporidiosis oocysts, the Modified Ziehl Neelsen (MZN) stain was prepared and samples were examined. The remaining two parts were immediately stored at -20°C for further assays.

Copro-immuno-detection of cryptosporidiosis: A complete set of the stored samples was subjected to *Cryptosporidium* coproantigen detection by using ELISA Kits (RIDASCREEN *Cryptosporidium*-C1201-R-Biopharm AG, Germany) by the specific antibodies to *C. parvum* and *C. hominis* detection. Processing was done according to the manufacturer's instructions.

Copro-PCR assay: nPCR technique was used for molecular diagnosis of cryptosporidiosis using *Cryptosporidium* oocyst wall protein (Spano *et al*, 1997). The last set of the stored samples was subjected to DNA extraction using Favor Prep DNA isolation Kit (Favorgen Biotech, Taiwan, Cat. No. FASTI001-1). Processing of samples was done according to the manufacturer's instructions.

PCR amplification: Performing of samples was done using two sets of primers; BCOWP forward and reverse (Pedraza-Díaz *et al*, 2000), mixture and conditions of reaction were done. PCR amplified products were run on agarose gel electrophoresis (1.5%) then visualized by the UV Transilluminator after adding the ethidium bromide stain.

Statistical analysis: By using the statistical package SPSS version 17 (Chicago, IL, USA). Considering nPCR as a gold reference standard, sensitivity, specificity, PPV and NPV were estimated and the kappa agreement was performed to prove validity of acid fast stained microscopy and ELISA regarding nPCR results in diagnosis of *Cryptosporidium* infection. Percentage and frequency were used to describe qualitative variables while $M \pm SD$ was designed for quantitative variables. Risk factors for acquiring infection diagnosed by nPCR were done using univariable logistic regression analysis. The odds ratio (OR), 95% confidence interval (CI) and *P* value (≤ 0.05) were estimated.

Results

Diagnostic validity: Out of 189 children, *Cryptosporidium* infection was proved molecularly in 13 samples (6.8%), by ELISA in 7.9% of samples and by stained microscopy in 3.7%. Microscopy versus ELISA gave maximum specificity and PPV (100% for each) than ELISA, which gave higher sensitivity (77%) than stool (53.8%). Both microscopy and ELISA gave a substantial Kappa agreement (0.61, 0.62, respectively) as diagnostic tools for cryptosporidiosis infection.

Cryptosporidiosis and associated variables: The mean age of infected children ($n=13$) was 2.9 ± 1.4 . Cryptosporidiosis was more prevalent among males, inhabiting rural areas, using tap water (61.5%, 69.2% & 69.2%, respectively). Infection was more common among children with preschool age (46.2%) followed by toddlers (38.5%) and finally infants (15.4%). Age groups were significantly associated with infection ($P = \leq 0.05$). Source of water and presence of undigested food particles on microscopy were risks for infections (P value ≤ 0.05). The diarrhea followed by abdominal pain and pyrexia were presented among infected children (76.9%, 30.8% & 23.1%, respectively). About 76.9% of the *Cryptosporidium* infected children harbo-

ured another parasite or commensal on their stool. Stool analysis of infected samples showed undigested food and fat particles (69.2% & 46.2%, respectively). Only

presence of undigested food in stool was predictive for infections ($P = \leq 0.05$).

Details were given in tables (1, 2 & 3) and figure (1)

Table 1: Microscopy, ELISA and nPCR

		Microscopy			ELISA		
		+ve	-ve	Total	+ve	-ve	Total
n PCR (n=13)	Positive	7 (53.8%)	6 (46.2%)	13 (6.8%)	10 (76.9%)	3 (23.1%)	13 (6.8%)
	Negative	0 (0%)	176 (100%)	176 (93.1%)	5 (2.8%)	171 (97.2%)	176 (93.1%)
	Total	7 (3.7%)	182 (96.3%)	189 (100%)	15 (7.9%)	174 (92%)	189 (100%)

Table 2: Diagnostic validity of stained microscopy and Ag detection by ELISA

Method	Sensitivity	Specificity	PPV	NPV	Kappa*
Microscopy	53.8 %	100%	100%	96.7 %	0.610
ELISA	77 %	97.1 %	66.6 %	98.2 %	0.624

*Kappa agreement interpretation: <0= poor, 0.01-0.20= slight, 0.21-0.40= fair, 0.41-0.60= moderate, 0.61-0.80 = substantial, and 0.81- 1.0= almost perfect

Table 3: Risk factors for patients with positive n PCR for cryptosporidiosis

Variables		+ nPCR n=13 (%)	-ve nPCR n=176 (%)	Total n=189 (%)	P value	95% C.I. (lower/upper)	OR	
sex	Male	8 (61.5)	80 (45.4)	88 (46.5)	0.2	0.604/6.101	1.920	
	Female	5 (38.5)	96(54.5)	101(53.4)				
Age years		2.9±1.4	3.2±2.7	3.1±1.9	0.07			
1. Infants (>1-2)		2 (15.4)	26 (14.7)	28 (14.8)	0.02*			
2. Toddlers (1-3)•		5 (38.5)	76 (43.1)	81 (42.8)				
3. Preschool (3-5)•		6 (46.2)	74 (42)	80 (42.3)				
Residence	Rural	9 (69.2)	135 (76.7)	144 (76.2)	0.3	0.428/4.999	1.463	
	urban	4 (30.8)	41 (23.2)	45 (23.8)				
Source of water	Tape	9 (69.2)	155 (82)	164 (86.7)	0.01*	0.004/1.165	0.069	
	Filtered	4 (30.8)	21 (11.9)	25 (13.2)				
Clinical manifestations	Diarrhea	Yes	10 (76.9)	103(58.5)		0.025/1.940	0.150	
		No	3 (23.1)	73(41.5)				76 (40.2)
	Abdominal pain	Yes	4 (30.8)	64 (36.4)	68 (35.9)	0.3	0.428/4.999	0.179
		No	9 (69.2)	112 (63.6)	121 (64)			
Fever	Yes	3 (23.1)	25(14.2)	28(14.8)	0.5	0.442/4.515	1.412	
	No	10 (76.9)	151(85.8)	165(85.2)				
Microscopic examination	Polyparasitism	Yes	10 (76.9)	103 (58.5)	0.1	0.469/6.323	1.790	
		No	3(23.1)	73(41.5)				76(40.2)
	Undigested food	yes	9 (69.2)	89(50.6)	98(51.8)	0.02*	0.521/6.154	1.058
		no	4 (30.8)	87(49.4)	91(48.1)			
	Fat particles	Yes	6 (46.2)	49(27.8)	55(29.1)	0.3	0.410/6.129	1.586
		No	7 (53.8)	127(72.2)	134(75.6)			
	RBCs	Yes	4 (30.8)	33(18.7)	37(19.6)	0.19	0.588/4.589	1.018
		No	9 (69.2)	143(81.3)	152(80.4)			
	Pus cells	Yes	5 (38.5)	38(21.6)	43(22.7)	0.17	0.293/1.297	1.418
		No	8 (61.5)	138(78.4)	146(77.2)			

•According to CDC, 2018, * significant P value (≤ 0.05).

Discussion

The present study revealed cryptosporidiosis infection among 6.8% of children. The

preschool children were the most affected (46.2%) followed by toddlers and infants (38.5% & 15.4%, respectively) with the age

group significant association ($P = \leq 0.05$). The children with preschool age used to play with pet animals, attend day care centers where wide range of risk factors for acquiring cryptosporidiosis are available at these communities at Egypt. Children are exposed to poor quality of water supply and infected children at nursery centers. One can't neglect the contaminated food when large number of children is on the responsibility of one person during feeding time. In addition to the poor personal hygiene for washing hands of children or their nanny during feeding and lack of following preventive tools by children to avoid infections at this age. These conditions express the high rate of infection at preschool children (Bouزيد *et al*, 2018; Ghieth *et al*, 2018). The toddlers were the second affected age group (38.5%); child at this age underwent a near social pattern to preschool age for attending day care center communities. In addition, toddlers are just start to be independent; they used to discover the world through sucking and tasting things, their toys and hands, an attitude making them vulnerable for infection.

In the present study, infants were the least affected group; two infants (15.4%) were *Cryptosporidium* positive by nPCR and were on bottle feeding, their mothers used to prepare milk formula by adding tap water to the milk powder. Also, they were complaining of diarrhoea.

During the first year, the child was more protected from *Cryptosporidium* especially those on exclusive breast feeding (Bouزيد *et al*, 2018). The fact that, breast milk shared nonspecifically by providing lactoferrin and specifically by IgA, that target the wall of *Cryptosporidium* oocyst improving protection against infection specially at endemic regions (Korpe *et al*, 2013). During the early 6 months; mothers milk protect infant by specific IgA against cryptosporidiosis, while during the weaning period the child is more exposed to acquire infection (Steinberg *et al*, 2004).

In Egypt, Naguib *et al*. (2018) found that

the frequent infections were at the ages of ≤ 3 & 4 years; El Matrawy *et al*. (2017) and Taha *et al*. (2018) reported the age range ≤ 3 to 9 with higher positivity for cryptosporidiosis. Besides, below the age of two; *Cryptosporidium* DNA product wasn't detected by nPCR (Taha *et al*, 2018). Similar results were recorded by Al-Shamiri *et al*. (2010) in Yemen children.

In contrast, others reported high infection rates below the ages of one (Bushen *et al*, 2007; Murugesan *et al*, 2017; Saha *et al*, 2019), and two (Hossain *et al*, 2019).

Dis-similarity between studies were in view of differences in the studied population, immunity, nutrition, time of weaning, either exclusive or non-breast feeding. Some mothers' traditional behaviors during the early childhood; forced the child to eat by his mother with bare hand increasing the chance for acquiring infection. Also the type of diet introduced to children with high carbohydrates and/or sugar low protein content, a diet pattern negatively affecting immunity and increasing inflammatory response.

Molecular diagnosis of cryptosporidiosis using nPCR was crucial (Ghieth *et al*, 2018), helped to detect infection among false negative cases by microscopy and ELISA. Microscopy betted on ELISA gave maximum specificity & PPV (100% for each), but some positive cases were missed due to low sensitivity (53.8%). The ELISA sensitivity (77%) was higher than stool. But, false positive cases and missed infections were restrictions (specificity 97.1%, & PPV 66.6 %).

The false negative results by ELISA may be by virtue of the used the monoclonal antibodies within the kits that tagged variable surface epitopes sets which may be not bind to all *Cryptosporidium* species. The persistence of antigenemia after clearance of infection or cross reactivity with other antigens boosts false positivity of ELISA (Ghoshal *et al*, 2018). In such a way, using nPCR alongside microscopy or ELISA is decisive for the diagnosis *Cryptosporidium* infection.

In the present study, infected children used

tape water (69.2%) and filtered water (30.8), source of water supply was a risk for infection (P value ≤ 0.05). *Cryptosporidium* oocyte was missed by chlorination; beside tape water in urban are stored over building tanks which can maintain infection if water is contaminated. *Cryptosporidium* is a small size 4-6µm water transmitted infection that escapes filtration devices, resists chemicals and being insistent in the environment (WHO 2006). In rural areas contamination of water surfaces, wells and stagnant water that used in domestic life are sources of oocyst. In addition to entertainment activities by children at early childhood age, and giving tape water to infants for drinking and preparing food could be sources of infection (Antonios *et al*, 2001).

In the present study, diarrhea was the most presented complains (76.9%), followed by abdominal pain and pyrexia (30.8%, 23.1%, respectively). Stool analysis of 76.9% of infected children was positive for one parasite or commensals, distributed as follow *Giardia intestinalis*, *Entamoeba* complex, *Entamoeba coli*, *Blastocyst hominis* (40%, 30%, 20%, & 10%, respectively). Since *Cryptosporidium* is acquired orally, it wasn't a surprise finding polyparasitism among children as they share the same route of infection (Ghieth, 2016; Moyo *et al*, 2017). This explained the variation of the clinical complains among the present studied populations. Stool analysis of infected samples showed undigested food and fat particles (69.2%, 46.2%, respectively), which was predictive for cryptosporidiosis ($P=0.02$).

Conclusion

Cryptosporidium is paediatric infection common among the preschool, toddlers and infants with the gastrointestinal manifestations especially diarrhea.

The source of water is suspected as risk for infection. Rate of filtered water transmitted cryptosporidiosis is lower than tape water.

The presence of the undigested food particles in stool predicated the cryptosporidiosis infection.

Consequently, the nPCR proved to be a good diagnostic tool to monitor *Cryptosporidium* infection.

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Explanation of figure

Fig.1: A- Agarose gel electrophoresis, L: DNA marker (100-bp); lane 1: positive control; lane 2: negative control; lanes 3-6: nPCR product at 553 bp targeting COWP gene of *Cryptosporidium*. B- *Cryptosporidium* oocyst stained with MZN stain, x100 oil immersion.

