Molecular genetic characterization of human *Cryptosporidium* isolates and their respective demographic, environmental and clinical manifestations in Egyptian diarrheic patients

Original Article

Adel G El-Missiry[1], Laila ME Abd El-Hameed[1], Ghada A Saad[1], Ayman A El-Badry[2], Yosra A Helmy[3,4], Mai A Shehata[1]

Medical Parasitology Department, Faculty of Medicine, Ain Shams[1] and Cairo[2] Universities, Cairo, Egypt; Food Animal Health Research Program, Department of Veterinary Preventive Medicine, Ohio Agricultural Research and Development Center, The Ohio State University; Wooster, USA[3]; and Department of Animal Hygiene, Zoonoses and Animal Ethology, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt[4]

ABSTRACT

Background: *Cryptosporidium* is a zoonotic protozoan parasite of public health and veterinary importance, causing gastroenteritis in a variety of vertebrate hosts. Several *Cryptosporidium* spp. with different genotypes and subtypes are implicated in human cryptosporidiosis.

Objective: Detection and correlation of native *Cryptosporidium* molecular genetic variability among Egyptian diarrheic human isolates with their respective demographics, environmental and clinical manifestations.

Patients and Methods: A total of 350 human stool samples were collected from diarrheic patients. The collected samples were examined microscopically and by RIDA®QUICK *Cryptosporidium/Giardia* copro-immunochromatography test (ICT) cassettes and strips. All positive *Cryptosporidium* samples detected by modified Ziehl-Neelsen stain (MZN) and/or ICT were genotyped using nested polymerase chain reaction-restriction fragment length polymorphism (nPCR-RFLP) targeting *cowp* gene encoding *Cryptosporidium* oocyst wall protein (COWP).

Results: *Cryptosporidium* was detected in 5.7% (20/350) of the examined samples using MZN, 9.4% (33/350) by ICT kit, and 3.7% (13/350) by both methods. A total of 40 samples were positive by both microscopy and/or ICT. Only 15/40 (37.5%) of samples positive by microscopy and/or ICT were successfully amplified using *cowp* gene. All amplified samples (15) were confirmed as *C. hominis* by nPCR-RFLP analysis of the *cowp* gene. Significant associations were found between cryptosporidiosis and gender, drinking groundwater, immunodeficiency of patients, complaining of fever, watery and mucoid stool, consumption of readymade salad, and unwashed fresh vegetables.

Conclusion: In the current examined samples, *C. hominis* proved to be the dominant genotype, indicating that transmission of cryptosporidiosis among a sample of diarrheic Egyptian patients is most probably anthroponotic rather than zoonotic. MZN and PCR gave a diagnostic accuracy of 100%, while ICT diagnostic accuracy proved to be 50%.

Keywords: *Cryptosporidium*, *C. hominis*, Egypt, nested PCR-RFLP, RIDA®QUICK *Cryptosporidium*/*Giardia*.

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Corresponding Author: Ghada A Saad, **Tel.:** 00201005818392, **E-mail:** dr_ghadasaad@yahoo.com

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INTRODUCTION

Cryptosporidium is a worldwide enteric zoonotic protozoan parasite infecting a broad range of hosts, including humans, ruminants, birds, reptiles and fish[1,2]. It is the main cause of diarrheal illness in both developed and developing countries[3]. Cryptosporidiosis can result in severe diarrhea, which is usually self-limited in immunocompetent individuals, but may be chronic and life-threatening in immunocompromised patients[4]. It was estimated that up to 10% of cryptosporidiosis occurred between one to nine years old children and in toddlers in developing countries[5]. It has been reported as a virulent cause of diarrhea in Egypt, especially in young children, with a varied prevalence of 0% - 49%[6,7]; and with a prevalence of 11.6% in symptomatic children in Qalubiyah province[8].

Humans can acquire *Cryptosporidium* infections by ingestion of infective oocysts through person-to-person transmission or by direct contact with livestock or *via* water or food-borne transmission[9]. Several *Cryptosporidium* species with different genotypes and subtypes may infect man. Each causative species has unusual source(s) of infection, transmission route(s), and pathogenicity. Up to date, more than 26 spp. and approximately 50 genotypes have been recognized in humans and animals[10]. Therefore, it was advised that species identification in populations is essentially vital for determining the local transmission risk factors, and the consequent implementation of control programs to limit contact with infectious oocysts[11]. The majority of human cases of cryptosporidiosis (more than 90%) are caused by two species: *C. hominis* and *C. parvum*[7,12]. However, other species including *C. felis, C. meleagridis,*

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C. canis, C. suis, and *C. muris* can also infect humans[13]. Of the two major genotypes that infect humans, *C. hominis* infections are specifically due to contact between humans only (anthroponotic infection), whereas *C. parvum* is a cause of zoonotic infections in humans and animals[14].

Diagnosis of cryptosporidiosis is generally based on microscopical detection of oocysts in stool, but this offers no information on the infecting species or subspecies[15]. Copro-immunochromatographic testing (ICT) is simple, rapid and offers a less subjective method than microscopy as it does not rely on microscopy skills. This technique was reported as a sensitive, specific, cost-effective, easy to perform, and rapid method[16].

An upgrade in diagnosis is by the use of new molecular genetics tools that allow species identification and subtyping of *Cryptosporidium*[4]. Polymerase chain reaction (PCR) is the most frequent diagnostic test and is also applied for genotyping with high diagnostic performance. Accordingly, PCR-RFLP analysis was implemented to understand the epidemiology and the study of disease outbreaks, using the gene encoding small subunit rRNA[17], and the *cowp* gene encoding the *Cryptosporidium* oocyst wall protein[18]. High prevalence rates previously reported from Egypt were recorded in rural areas where there is close contact between humans and animals. Between 1989 and 2016, the prevalence rates ranged between 3% and 50% in immunocompromised patients and up to 91% in diarrheic children in most of the Egyptian provinces[7]. In our present study, we aimed to correlate native *Cryptosporidium* molecular genetic variability among human isolates in a sample of diarrheic patients in Egypt, with their respective demographics, environmental and clinical manifestations. Evaluation of tested samples was based on microscopic, ICT and PCR identifications.

MATERIAL AND METHODS

A cross-sectional study was performed on fecal samples collected during the period between June 2013 and March 2015. Coproscopy and immunoassay were carried out in the Parasitology Diagnostic Unit, Parasitology Department, Faculty of Medicine, Ain Shams University, Egypt. Copro-nested (n) PCR-RFLP assay was performed in Molecular Medical Parasitology Lab., Department of Medical Parasitology, Faculty of Medicine, Cairo University, Egypt.

Samples collection: A total of 350 human diarrheic fecal samples were collected from patients attending the outpatient clinics of El-Demerdash hospitals (Pediatrics, Internal Medicine, Oncology), Parasitology Diagnostic Unit, Parasitology department, Faculty of Medicine, Ain Shams University, Fever hospital in El-Abbasyia (inpatient), Kasr Al-Ainy hospitals (Abu El Rish Pediatric hospitals and Internal Medicine, Cairo University) and Benha hospitals (Pediatrics, Internal Medicine, Benha University). The patients were of different ages: 214 patients were ≤ 5 years, 136 were ≥ 5 years; of both sexes, and different immune status. A predesigned questionnaire containing demographic, clinical and environmental data such as the source of drinking water, animal contact, water contact and history of consumption of ready-made and fresh unwashed vegetables was fulfilled for each patient.

Fecal samples: Fresh samples were collected in 60 ml clean, labeled containers. Each sample was divided into three portions; a small part for direct smear examination, another part was preserved in tight containers using 10% formalin for coproscopic examination and staining, and the third portion was stored at -20°C in Eppendorf tubes for immunoassays and molecular studies.

Coproscopic examination: All collected samples were examined microscopically using direct wet smear, formalin-ethyl acetate sedimentation concentration technique and MZN stain[19].

Coproimmunoassay: All fecal samples whether fresh or frozen were examined for detection of *Cryptosporidium* coproantigen using two ICT procedures[20,21]; RIDA®QUICK *Cryptosporidium*/ *Giardia* ICT cassettes and strips (R-Biopharm AG, Germany). These quick immunochromatographic tests qualitatively determine presence of *Cryptosporidium* and/or *Giardia* in stool samples, and were used to complement positive samples detected by microscopy. The test procedure was performed according to the manufacturer`s instructions. Briefly, 100 µL of each liquid or 50 mg of each solid fecal sample was diluted with one ml of extraction buffer in test tubes. The sample was then well homogenized and mixed either by up and down pipetting or by vortexing. The homogeneous suspension was left for at least 3 min to settle down forming a clear supernatant from which 200 µL was pipetted into the circular port of the test cassette. For the dipstick, 0.5 ml of the clear supernatant was pipetted into a new test tube and the strips were left in the supernatant for 5–10 min. Positive samples for *Cryptosporidium* gave a blue color band which appears at the same time as the green-colored control band[21].

Genomic DNA extraction from fecal samples: DNA extraction from the fresh and preserved samples was performed using the QIAamp Stool Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. This kit is designed for rapid purification of total DNA from up to 220 mg stool and is suitable for both fresh and frozen samples. Briefly, extraction of DNA was done by lysis of samples in the fecal lysis buffer. One Inhibit EX tablet was added to each sample and vortexed immediately continuously for 1 min until the tablet was completely suspended. The suspension

was then incubated for 5 min at room temperature to allow inhibitors to adsorb to the Inhibit EX matrix. Purification of extracted DNA was done on QIAamp spin columns. DNA was then eluted in 50 µl buffer (Qiagen) and stored at -20°C till use.

Amplification of extracted genomic DNA (Copro-n-PCR): For the molecular characterization of *Cryptosporidium* spp., amplification of the extracted genomic DNA was performed using nPCR analysis of *cowp* gene. Amplification was done in accordance to the manufacturer's instructions. Two sets of primers were used such that the first set was used to produce an amplicon template for the nested reaction[22]. The second set of primers was used to anneal internally within the previously obtained amplicon increasing the specificity of detection[23]. The details of the target gene, primers sequences, and amplicon size are shown in table (1).

Restriction fragment length polymorphism (RFLP): A conventional PCR-RFLP assay was conducted to differentiate *Cryptosporidium* spp.[24] by digestion of the amplified nPCR products. The DNA sample was digested by RsaI restriction enzyme (Thermo Scientific #FD1124) and the resulting restriction fragments were separated according to their lengths by gel electrophoresis. Restriction assays were performed in 30 µL volume with 2 units of restriction enzyme and 5 µL of PCR product per reaction. Mixtures were incubated in a heating block. Digestion products were visualized under UV light in 2% agarose gel electrophoresis after ethidium bromide staining. Digested PCR product with these enzymes resulted in a distinct band pattern for the human and bovine genotypes. The amplicon size of the digested product for *C. parvum* and *C. hominis* was 220 bp and 266 bp, respectively.

Statistical analysis: Mean, standard deviation (±SD) and range were used for parametric numerical data, while median and interquartile range (IQR) were used for non-parametric numerical data. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and diagnostic accuracy were calculated[25]. Statistical analysis was performed using statistical package for Social Science (SPSS) software. *P* value <0.05 was considered statistically significant.

Ethical approval: The research protocol was approved by the Ethics Committee, Faculty of Medicine, Ain Shams University as it complies with the 1964 Helsinki declaration. Verbal consent from participants or from parents or guardians of children in the study was obtained after a clear explanation of the study objectives. Patients infected either with *Cryptosporidium* or other parasites were prescribed suitable treatment.

Table 1: Primers used in PCR for the target *cowp* gene and its sequence[22].

Primers	Sequence	Product size	Annealing temperature
Primary	1) Forward primer (COWP-F): 5'-ACCGCTTCTCAACAACCATCTTGTCCTC-3' 2) Reverse primer (COWP-R): 5'-CGCACCTGTTCCCACTCAATGTAAACCC-3'	769 bp	63°C
Secondary	1) Forward primer (Cry-15): 5'-GTAGATAATGGAAGAGATTGTG-3' 2) Reverse primer (Cry-9): 5'-GGACTGAAATACAGGCATTATCTTG-3	553 bp	54°C

RESULTS

Microscopic examination: Using MZN stained fecal smear, 20/350 samples (5.7%), were positive for *Cryptosporidium* oocysts.

Crypto-*Giardia* ICT (Figure 1): Using the Crypto-*Giardia* ICT, 33/350 (9.4%) and 21/350 (6%) fecal samples were immune reactive for *Cryptosporidium* and *G. duodenalis*, respectively. Additionally, 9/350 (2.6%) samples were co-infected with both *Cryptosporidium* and *G. duodenalis*. The Crypto-*Giardia* ICT kit confirmed only 13 (65%) positive samples out of the 20 *Cryptosporidium* positive samples diagnosed by MZN stain (Tables 2, and 3).

Nested-PCR amplification of *cowp* gene (Figure 2): DNA was extracted from 40 stool samples that were all positive for *Cryptosporidium* either by microscopic examination, or by Crypto-*Giardia* ICT kit, or by both methods. Using *cowp* gene, 15/40 stool (37.5%) samples were successfully amplified at 553 bp.

Fig. 1: RIDA®QUICK Crypto-*Giardia* ICT cassette [A] and strips [B]
(a) Negative test with green control band only.
(b) Positive *G. duodenalis* test showing 2 bands; control green band and test red band of *G. duodenalis*.
(c) Positive *Cryptosporidium* test showing 2 bands; control green band and test blue band of *Cryptosporidium*.
(d) Positive mixed *Cryptosporidium* and *G. duodenalis* test showing 3 bands; control green band, test red band of *G. duodenalis* and blue band of *Cryptosporidium*.

Table 2: Results of MZN stain, ICT vs nPCR in detection of cryptosporidiosis in 40 positive cases.

Diagnostic test		nPCR					
		Negative		Positive		Total	
		No.	%	No.	%	No.	%
MZN	Positive	20	50.0	0	0.0	20	50.0
	Negative	5	12.5	15	100.0	20	50.0
ICT	Positive	5	12.5	2	5.0	7	17.5
	Negative	20	50.0	13	32.5	33	82.5
Total		25	62.5	15	37.5	40	100.0

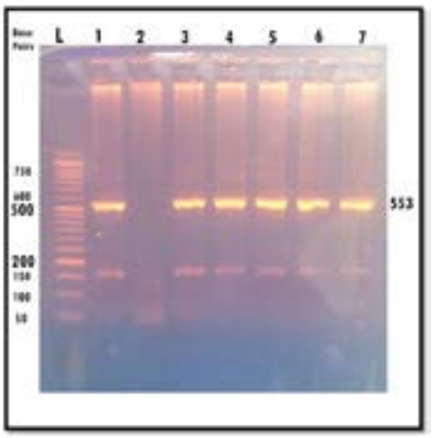

Fig. 2: An ethidium bromide stained agarose gel electrophoresis showing the secondary PCR products of *Cryptosporidium cowp* gene (553 bp). **L:** DNA molecular weight marker (50 bp), **Lane 1:** Positive control, **Lane 2:** Negative control, **Lanes 3-7:** Positive samples

Table 3. *Cryptosporidium* positive samples with different methods used for diagnosis in 40 positive cases by both microscopy and/or ICT vs nPCR.

No.	MZN	ICT	nPCR	+ve/-ve	No.	MZN	ICT	nPCR	+ve/-ve
1	+	-	-	+	**21**	-	+	-	+/-
2	-	+	-	+/-	**22**	+	+	+	+
3	-	+	-	+/-	**23**	+	+	+	+
4	-	+	-	+/-	**24**	+	+	+	+
5	+	-	-	+	**25**	-	+	-	+/-
6	-	+ Crypto-*Giardia*	-	+/-	**26**	-	+ Crypto-*Giardia*	-	+/-
7	-	+	-	+/-	**27**	-	+ Crypto-*Giardia*	-	+/-
8	+	-	-	+	**28**	+	+	+	+
9	-	+	-	+/-	**29**	-	+	-	+/-
10	+	-	+	+	**30**	+	+	+	+
11	-	+	-	+/-	**31**	+	+ Crypto-*Giardia*	+	+
12	-	+ Crypto-*Giardia*	-	+/-	**32**	-	+	-	+/-
13	-	+	-	+/-	**33**	-	+ Crypto-*Giardia*	-	+/-
14	+	-	-	+	**34**	-	+	-	+/-
15	+	-	+	+	**35**	-	+	-	+/-
16	-	+	-	+/-	**36**	+	+ Crypto-*Giardia*	+	+
17	+	-	-	+	**37**	+	+	+	+
18	+	+	+	+	**38**	+	+	+	+
19	+	+	+	+	**39**	-	+	-	+/-
20	+	+ Crypto-*Giardia*	+	+	**40**	+	+ Crypto-*Giardia*	+	+

+/- The sample is either true positive or true negative.

Diagnostic yield of the used techniques: Twenty out of the 40 samples (50%) gave positive results for cryptosporidiosis using MZN, among them 5 samples (12.5%) were negative by PCR. As regards ICT, 33 out of the 40 samples gave positive result; 13 of them (82.5%) were also positive by MZN and PCR and the remaining 20 samples (50%) were considered either positive or negative (+/-). The last 7 samples (17.5%) positive by ICT gave negative results; despite being positive by MZN, 2 of them (5%) were positive by PCR (Table 3). Accordingly, the calculated sensitivity, specificity, PPV, NPV and diagnostic accuracy for the used techniques showed 100% for each parameter by MZN and PCR. By ICT the parameters were 65%, 48%, 36%, 59%, respectively (Table 4).

Risk factors for contracting *Cryptosporidium* **infection:** The potential risk factors were interpreted and the relationship between them and the detection rate of cryptosporidiosis for all the detected positive samples by MZN and nPCR, are presented in table (5). The results revealed a high association between the detection rate of cryptosporidiosis and gender of the patients. A significantly higher ($P \leq 0.05$) detection rate of infection was recorded in males than females, among patients drinking groundwater than those drinking tap water, in immunodeficient patients compared to immunocompetent patients, and among patients suffering from fever in comparison to those with no fever.

Furthermore, the detection rate of *Cryptosporidium* was significantly higher ($P \leq 0.05$) in patients with watery and mucoid stool than that in patients with watery stool, and in patients eating readymade salad and unwashed fresh vegetables than that in their counterparts. There was no apparent influence of age,

residence, contact with animals, localities, and contact with water on the detection rate of cryptosporidiosis; but the it was higher in summer (50%) compared to spring (30%).

Molecular characterization of *Cryptosporidium* **by n-PCR-RFLP:** Results proved that *C. hominis* is the only detected species. No other *Cryptosporidium* spp. was detected (Figure 3).

Table (4): Diagnostic yield of MZN stain, ICT and nPCR.

	Diagnostic techniques		
	MZN	**ICT**	**nPCR**
Sensitivity%	100	65	100
Specificity%	100	48	100
Positive predictive value%	100	39	100
Negative predictive value%	100	26	100
Diagnostic accuracy%	100	50	100

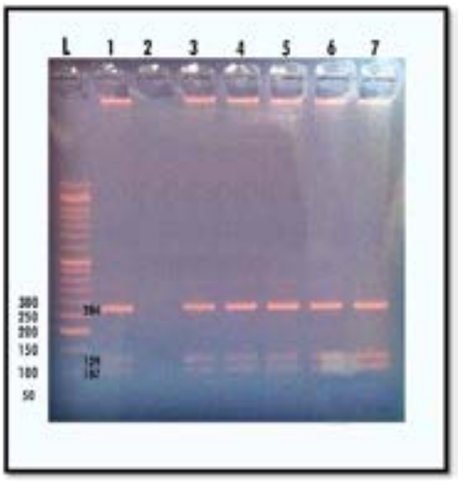

Fig. 3: *C. hominis* bands at 284,129 and 107 bp molecular weight by RFLP in comparison with the reference strain. **L:** 50 bp DNA molecular weight marker, **Lane 1:** Positive control, **Lane 2:** Negative control, **Lanes 3-7:** Positive samples

Table (5). Analysis of potential risk factors for cryptosporidiosis among patients enrolled in the study.

Risk factor		**MZN**		**ICT**		**nPCR**	
Variable (No.)		**No. (%)**	**Statistical Analysis**	**No. (%)**	**Statistical Analysis**	**No. (%)**	**Statistical Analysis**
Age	<5 (27)	13 (48.1)	CI: 30.7- 66.0	23 (85.1)	CI: 66.9-94.7	11 (40.4)	CI: 24.5-59.3
	> 5 (13)	7 (53.8)	29.1-76.8	10 (76.9)	42-87.6	4 (30.8)	12.7-57.6
Sex	M (26)	**16 (61.5)***	42.4-77.6	21 (80.7)	61.6-91.9	**13 (50) ***	32.1-67.9
	F (14)	**4 (28.5)***	11.3-55.0	12 (85.7)	51.6-93.1	2 (14.3)	4.0-39.9
Residence	City (20)	8 (40)	21.8-61.4	16 (80)	57.8-92.5	5 (25)	11.2-46.9
	Village (20)	12 (60)	38.6-78.1	17 (85)	57.8-92.5	10 (50)	29.9-70.1
Drinking water	Tap (26)	**10 (38.4)***	22.3-57.5	20 (76.9)	57.6-89.3	**6 (23.1) ***	11-42.1
	Ground (14)	**10 (71.4)***	44.6-88.6	13 (92.8)	58.8-97.2	**9 (64.3) ***	38.8-83.7
Animal contact	Negative (26)	11 (40.7)	24.4-59.3	21 (77.8)	55.0-87.0	**7 (25.9) ***	13.2-44.7
	Positive (14)	9 (69.2)	42.0-87.6	12 (92.3)	64.5-99.9	**8 (61.5) ***	35.5-82.3
Immuno-deficiency	Negative (21)	**4 (19)***	7.0-40.5	16 (76.1)	54.5-89.7	**1 (4.8) ***	0.8-22.7
	Positive (19)	**16 (84.2)***	61.6-95.3	17 (89.5)	61.6-95.3	**14 (73.7) ***	51.2-88.2
Water contact	Negative (30)	14 (46.7)	21.8-61.8	26 (86.7)	59.8-92.5	10 (33.3)	11.7-57.6
	Positive (10)	6 (60)	42.9-77.6	7 (70)	43.9-87.6	5 (50)	31.1-67.8
Stool consistency	W (14)	**4 (28.6)***	11.3-55	11 (78.6)	42.0-87.6	1 (7.1)	2.5-25.4
	W & M (26)	**16 (61.5)***	38.6-78.1	22 (84.6)	57.8-92.5	14 (53.8)	32.1-67.9
Fever	Negative (12)	**5 (41.7)***	41.7	10 (83.3)	57.8-92.5	**2 (16.7) ***	5.0-39.9
	Positive (28)	**15 (53.6)***	53.6	23 (82.1)	43.9-87.6	**13 (46.4) ***	30.5-59.3
Eating vegetables	Negative (19)	**3 (15.8)***	15.8	13 (68.4)	45.7-97.3	2 (10.5)	30.0-44.2
	Positive (21)	**17 (81)***	81.0	20 (95.2)	75.3-98.0	13 (62)	38.8-83.7

M: male, **F:** female, **W:** watery, W & M: watery and mucoid
*** *P* value < 0.05.**

DISCUSSION

In the present study, the *Cryptosporidium* infection rate of 5.7% recorded by microscopic examination of MZN stained smears of all fecal samples, is consistent with a previous rate of 4.6% reported from ten public hospitals in Cairo Governorate[26]. Moreover, another study done on Egyptian diarrheic children attending Cairo University hospitals reported a prevalence of 7.4%[27]. However, 37.7% and 91% were generally recorded in immunodeficient children and adult patients respectively in Egypt[28]; as well as 33.3% in immunocompromised children in Tanta Governorate[29] and 15% in El-Sharkiya Governorate[30]. The discrepancy in infection rates is expected due to the influence of immune state and age of patients, environmental habitats or seasonal variation[31,32], sample size and virulence of different isolates of the parasite[33,34] .

Our study showed that sensitivity and specificity, PPV, NPV of microscopical examination using MZN stain was 100%. A previous study[35] certified that MZN stain sensitivity was 83.7% and specificity 98.8%. Other studies[36,37] reported that MZN stain gave a lower sensitivity and specificity of 55.3% and 79%, respectively. Results by MZN staining may be hindered by the need for three consecutive stool samples examination, and technical experience with the concentration and staining method[38], as well as the possible microscopic misidentification of *Cryptosporidium* as *Cyclospora*[35]. In our study, no false positive results were detected by MZN stain microscopic examination. Confirmation of cryptosporidiosis using RIDA Crypto-*Giardia* ICT kit showed that out of the 350 samples examined in our study only 9.4% (33 samples) were immune reactive for *Cryptosporidium*. Thirteen of the samples were considered true positive as they were also positive by MZN and PCR, while 20 samples were regarded as true or false positive because they were negative using both MZN and PCR techniques. False positive may be due to cross reaction with other antigens[21]. In two previous studies[20,39] *Cryptosporidium* oocysts were detected in 15.3% and 13.6% of samples respectively by the same test. While higher records were documented in 89.5% of cases in a study in Suez Canal, Ismailia, Egypt[37], a much lower immune-reactive result of 5.88% was reported with ICT in Cairo. ICT rates recorded in our study were 65% sensitive, 48% specific, with 39% PPV and 26% NPV and a diagnostic accuracy of 50%. A previous study evaluated different copro-diagnostic tests for the detection of *Cryptosporidium* and concluded that although less time-consuming and easier to perform, they were less sensitive than conventional microscopic methods[40]. In another report, when compared to staining the immune-card test recorded a sensitivity of 86.7% and specificity 100%[41]. Such conflicting evaluations reduce the advantage of rapid detection of *Cryptosporidium* by ICT immunoassay, which may be explained by the persistence of antigens for several days after treatment, or an abortive infection, or by cross-reaction with other antigens[42]. It was reported that several mAbs against oocysts antigens of *C. parvum* cross react with other parasitic life cycle stages, other *Cryptosporidium* species or other coccidian parasites[43,44]. Because reaction of the immuno-card test may persist after the patient stops shedding intact organisms, our results may not be false-positive but may rather represent recently cured cases[45]. On the other hand some positive-microscopy samples with low parasitic load were reportedly negative by ICT[26]. This may explain the false negative results in our study.

Only those fecal samples positive by microscopy and/or Crypto-*Giardia* ICT kit methods (40 samples) were subjected to DNA extraction. Using the nPCR targeting *cowp* gene, 15/40 (37.5%) of positive samples by MZN and 13/40 (32.5%) of positive samples by Crypto-*Giardia* ICT kit were successfully amplified. However, 5 (12.5%) of positive samples by MZN and 20 (50.0%) of positive samples by ICT were not amplified. According to previous evaluations we validated the diagnosis of cryptosporidiosis by considering the detection of the organism by microscopy and or PCR as true positive cases[46] where a total of 15 samples gave positive results by microscopy were confirmed by PCR which showed the highest sensitivity and specificity of 100% respectively. Previously, it was reported that the sensitivity and specificity of the PCR method was 83.3% and 95.8% respectively[47]. In the literature researchers explained lower positivity found by PCR as opposed to microscopy due to the presence of few oocysts that were not distributed homogeneously within the fecal sample. Authors claimed that their results and those of previous studies indicated that the detection of *Cryptosporidium* oocysts in fecal smears by microscopy is less sensitive than the detection of DNA by PCR[48]. However, two other studies[46,49] reported 100% sensitivity and specificity in the detection of *Cryptosporidium* by PCR. Another study reported a positive result of 75% (9/12) by nested PCR of *cowp* gene in a positive sample of diarrheic children from the Gastroenteritis Unit, Abo El Reesh Hospital, Cairo University[50].

Among the variables in our study, the difference in sex distribution was statistically significant where *Cryptosporidium* was detected more in males, which is in accordance with results recorded previously[51-53] and is attributed to the possible fact that males are more active, mobile, and in contact with environmental activities. In relation to residency, the frequency of infection was more pronounced in those from villages than in the city, which again agrees with other reports[39,51,53-55]. Generally the high infection rate in rural areas is attributed to the difference in lifestyle due to low socioeconomic conditions and poor hygienic behavior, type of feeding, overcrowding, and contact with animals and water source. Ground water proved to be the main source of drinking water in some positive

cases in our study. Together with previous similar results[39,56] the threat of *Cryptosporidium* in water supplies is confirmed and attributed to resistance of its infecting stages to chlorine disinfection[57]. Cryptosporidiosis in both adults and children has been linked to improperly treated drinking water or exposure to contaminated recreational water[58]. The lack of proper sanitation and infrastructure may be the origin of water contamination by fecal materials or they may become contaminated by storage in unclean containers[59]. We found no significant association between *Cryptosporidium* infection and history of animal or water contact. Similarly, other reports[7,27] failed to find animal association with *Cryptosporidium* infection. In a recent report on occupational infection hazards in sewage workers in Alexandria, Egypt, among other protozoa *Cryptosporidium* rated 15.6%[60].

As expected there was a significant association between *Cryptosporidium* infection and the immune status of patients, with a higher prevalence among those who were immunosuppressed. when compared to those who were immunocompetent. A previous study[61] included immunosuppressed individuals in groups known to be highly vulnerable to opportunistic infections such as cryptosporidiosis. Our patients with complains of fever showed a significantly higher detection rate of *Cryptosporidium* infection than those who had no fever. A previous study[62] supported our findings and attributed fever to the secretion of the cytokine interleukin-1 (IL-1) by the host cells in response to the presence of the parasite.

Also, stool consistency in the present study was significantly associated with *Cryptosporidium* infection. Among our studied group of patients, watery mucoid stool was of higher prevalence than watery stool. Likewise, other reports corroborated our results[39,62,63] and deduced that infection induces defects in intestinal permeability. It was explained that the increased permeability may result in decreased absorption of fluids and electrolytes as well as diminution of solute fluxes into the gut. While only watery stool was reported by other studies[21,53]. In addition we recorded a significant association between consuming ready-made salad from the market and unwashed fresh vegetables with the occurrence of cryptosporidiosis. These results agreed with other announcements[55,61] confirming *Cryptosporidium* infection due to the consumption of raw vegetables. The usage of human excreta as manure in crops, open defecation by children in fields and free grazing of cattle along riversides may lead to contamination of river water subsequently used to irrigate fields and for washing the harvest[64]. Most of the *Cryptosporidium* cases in our study were detected during summer mainly in August (10 cases), and this agreed with other studies from different regions in Egypt[26,27,36,56]. The summer seasonal diarrheal peak may coexist with the increased use of rivers, lakes, swimming pools and water parks[65]. Also, there was a second rise in infections in May (3 cases) in our study. Seasonal variation has been related to the cycle of transmission and to *Cryptosporidium* species involved[66].

Results obtained by applying PCR-RFLP of the *cowp* gene using Rsa1 restriction enzyme indicated that *C. hominis* was the only species identified in our study. This was in accordance with several previous studies[27,67,68], as well as with another report specifying that *C. hominis*, was the only genotype detected among diarrheic children in Cairo, Egypt[50]. Other results with *C. hominis* genotype predominance recorded an outcome of 80% for *C. hominis* and 40% for *C. parvum*[26]. Also *C. hominis* was reported as 1.6 times more prevalent than *C. parvum* in children in Ismailia, Egypt[5]. Furthermore, 15 vs 5 *C. hominis* and *C. parvum* respectively was recorded[67]. This result was attributed to contamination of surface water by human sewage rather than contamination from an animal source.

The variation in *Cryptosporidium* genotypes distribution was accredited to differences in the impact of infection sources. The peak of *Cryptosporidium* bovine strain was attributed to land use patterns of polluted water supplies by young livestock (main reservoir), by hydrological means in areas with rainfall/flood actions; as well as the farming practices related to calving. On the other hand human strain predominance was linked to water contamination from human activities and with person-to-person transmission[70].

In conclusion, identification of the *cowp* gene using Rsa1 restriction enzyme showed that *C. hominis* was the only species recognized in our study. Influence of population dynamics was signified by the prevalence of anthroponotic *Cryptosporidium* strain and revealed the transmission pattern of *Cryptosporidium* among the studied group of patients. Water pollution may be the explanation of distinctive seasonality of this species in Egypt together with people dynamics that lead to increased person to person transmission in hot months and spring due to increased outdoor actions. The lack of appropriate sanitation and infrastructure may be the origin of water contamination by fecal materials or they may become polluted by storage in infected containers. Regarding diagnostic tests used in this study, MZN and PCR gave a diagnostic accuracy of 100%, while ICT diagnostic accuracy was found to be 50%.

Authors Contribution: El-Missiry AG shared in designing the plan of work and revising the manuscript; Abd El-Hameed LM conceived and designed the plan of work, analyzed the data and revised the manuscript; Saad GA shared in performing the staining, microscopic examination and wrote the manuscript; El-Badry AA shared in performing the molecular part of the study; Helmy YA shared in immunochromatography test and revised the manuscript; Shehata MA collected the samples, took history and filled the questionnaire

from patients, shared in the staining and microscopic examination.

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