Original Article

Detection rate and genotyping of *Cryptosporidium* spp. and its relation to copro TNF-α in elderly Egyptians attending outpatient clinics of Cairo University Hospitals

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ABSTRACT

Background: Elderly individuals are considered an at-risk population, susceptible to enteric infections; and *Cryptosporidium* spp. is an apicomplexan protozoan considered to be one of the most common protozoa causing diarrhea. Cryptosporidiosis causes elevation of many pro-inflammatory cytokines like tumor necrosis factor-alpha (TNF-α) which may play a role in pathogenesis of the disease.

Objectives: This study was designed for detection and genotyping of *Cryptosporidium* spp. in elderly patients and the relationship of infection with copro TNF-α. Diagnosis was by evaluation of permanent acid-fast cold Kinyoun's (AF) staining, immunochromatographic detection (ICT), and ELISA in comparison to molecular diagnosis as gold standard diagnostic method.

Subjects and Methods: Stool samples were collected from 270 elderly patients aged above 60 years old attending outpatient clinics of Internal Medicine Hospital, Cairo University. Samples were examined microscopically by direct wet mount, and AF staining, and then subjected to ICT, ELISA, and nested PCR (nPCR) assays. Positive samples by nPCR were then subjected to Restriction fragment length polymorphism (RFLP) to detect *Cryptosporidium* genotypes. Copro-levels of TNF-α were measured to assess their relationship with cryptosporidiosis.

Results: Cryptosporidiosis detection rates of 3.7%, 6.3%, 6.7%, 3.7% were determined by microscopic examination after AF staining, ICT, ELISA and nPCR, respectively. When RFLP was performed on nPCR positive samples, eight and two samples were assigned as genotype 1 and 2, respectively. Moreover, TNF-α was significantly correlated with cryptosporidiosis.

Conclusion: The elderly are highly vulnerable to cryptosporidiosis. Immunodiagnosis and molecular techniques are fundamental for the diagnosis of cryptosporidiosis. Cryptosporidiosis significantly affects copro TNF-α.

Keywords: AF stain, elderly, Cairo, *Cryptosporidium* spp., ELISA, ICT, nested PCR.

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INTRODUCTION

Cryptosporidium spp. are apicomplexan protozoa causing intestinal disorders including diarrhea in humans worldwide[1]. Infection can be transmitted mainly by the consumption of contaminated food and water containing the infective oocysts[2]. The symptoms of acute infection with cryptosporidiosis involve severe watery diarrhea, nausea, abdominal pain, low-grade fever, malabsorption, malnutrition; leading to dehydration[3-5]. The disease is usually self-limited in immunocompetent individuals and proceeds to chronic and life-threatening conditions in immunocompromised patients[6]. The elderly population may be particularly sensitive to cryptosporidiosis and this may be attributed to changes in the immune system and gastrointestinal functions that occur with aging, leading to increased susceptibility to enteric infections[7,8]. Since 1998, the recorded annual rate of cryptosporidiosis in the United States among persons aged ≥65 years varied between 0.29 and 0.70 cases per 100,000 populations. This minimal number of cases is attributed to the non-obligatory reporting to the CDC[9]. Moreover, *Cryptosporidium* spp. are considered to be a main cause of waterborne outbreaks worldwide, and there are 239 waterborne outbreaks reported between 2011 and 2016[10]. Thus, in 2004, the WHO listed it among the globally "neglected diseases", linked with poverty in most developing countries[11].

Cryptosporidiosis is mainly diagnosed by microscopic detection of oocysts in stained stool smears[3]. To facilitate and improve testing, copro-antigen commercial tests, such as ICT, and ELISA were used, because they are rapid, simple, cost-effective and don't depend on microscopy skills[12,13]. Later, molecular techniques including polymerase chain reaction (PCR) based assays have become reference methods that offer sensitive and specific diagnosis of *Cryptosporidium* spp.[14], in addition to identification of infecting species[6]. Concerning this issue, several molecular studies have revealed that different species of *Cryptosporidium* may infect man. Currently, more than 26 species and nearly 50 genotypes have been documented in humans and animals[15]. The human

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cases of cryptosporidiosis are mainly caused by two species: *C. parvum* and *C. hominis*[16]. However, other species as *C. felis, C. canis, C. meleagridis, C. muris,* and *C. suiscan* also infect humans[17].

It was observed that cryptosporidiosis correlates with the elevation of many pro-inflammatory cytokines such as TNF-α and interleukin (IL)-1β; these cytokines are key stimulators of prostaglandin synthesis[18]. In the intestinal wall, prostaglandins are expressed in response to these pro-inflammatory cytokines and may contribute to diarrhea by altering chloride secretion. Thus, TNF-α, IL-1β and prostaglandins have been assumed to have a role in the pathogenesis of cryptosporidiosis[19].

The aim of our study is to relate cryptosporidiosis in elderly patients to copro TNF-α. Simultaneously, we compared the diagnostic sensitivity and specificity of the AF stain, and the commercially available copro-antigen tests; *Cryptosporidium* ELISA, and RIDA® QUICK-ICT against PCR as the reference method[20,21].

SUBJECTS AND METHODS

This descriptive analytical study was conducted at Medical Parasitology, and Clinical Pathology departments, Cairo University, Faculty of Medicine during the period from May 2019 to June 2020.

Subjects: The study included 270 elderly diarrheic patients who were selected from Internal Medicine Hospital, outpatients' clinic, Cairo University. Inclusion criteria include both sexes, aged above 60 years, with diarrhea. The patient was considered diarrheic when complaining of abnormal increase in stool liquidity and frequency of bowel motions. Patients were subjected to thorough history taking including type of drinking water, level of education, residence, vomiting, history of hepatitis C, and abdominal pain, as well as clinical examination.

Stool samples collection: A single fecal sample from each patient was collected in a dry, clean, leak-proof plastic container. The macroscopic examination of the fecal samples was recorded before processing. Each sample was divided into 2 parts; fresh and frozen at -20˚C.

I. Detection rate of cryptosporidiosis

1. **Microscopic examination:** The stool samples were first examined by direct wet mount after the addition of a drop of iodine, and by formalin-ethyl acetate sedimentation techniques to aid visualization of protozoa cysts, helminthes eggs and larvae.

2. **Permanent cold AF Kinyoun's staining:** Thin stool smears were prepared from the sediment obtained by the formalin ethyl acetate sedimentation procedure and stained with cold AF stain for detection of oocysts. A commercially available readily prepared AF stain was used (Kinyoun Kit cat no 25765-1, Polysciences, Germany). Oval/round bodies, about 4-5 µm, of *Cryptosporidium* oocysts stained pink to red to deep purple, often with darker staining around the periphery, were identified against a blue background. In some of the oocysts, four sporozoites were visible[22].

3. **Immunochromatographic test (ICT):** Fresh samples were also processed using RIDA® QUICK *Cryptosporidium* test (Biopharm, Germany) for the qualitative determination of *C. parvum* in stool samples. This test is a fast single-step ICT in which the sample was positive if the red and blue bands are visible, the sample was negative if only the blue band is visible and not valid if there were no visible bands. Likewise, changes in band color that appear after 10 minutes or later were also without any diagnostic value and not used for evaluation.

4. **Enzyme linked immunosorbent assay (ELISA):** Part of each frozen fecal specimens was processed for detection of *Cryptosporidium* copro-antigen using RIDASCREEN® *Cryptosporidium* ELISA (*Cryptosporidium*-C1201, Germany) according to the manufacturer's instructions[23]. In order to establish the cut-off point, extinction units (0.15) were added to the measured extinction for the negative control. The specimen was considered positive if the extinction rate was 10% higher than the calculated cut-off value, while samples with extinctions 10% less than calculated cut-off were considered negative.

5. **Nested PCR (nPCR)[24,25]:** In this study, nPCR was considered the gold standard method for diagnosis of cryptosporidiosis[20,21]. Genomic DNA extraction from stool samples was done using FavorPrepTM

Table 1. The thermocycler program for nPCR reactions.

Steps	Process	Primary reaction		Secondary reaction		Cycle number
		Temp.	Time	Temp.	Time	
1	**Initial denaturation**	95°C	4 min	95°C	4 min	1
2	**Denaturation**	94°C	1 min	94°C	50 sec	35
3	**Annealing**	63°C	1 min	54°C	30 sec	35
4	**Extension**	72°C	1 min	72°C	50 sec	35
5	**Final extension**	72°C	10 min	72°C	10 min	1
6	**Infinite**	4°C	∞	4°C	∞	--

stool DNA isolation Mini Kit (Cat. No. FASTI 001, Favorgen Biotech corporation Ping-Tung, Taiwan). Extracted copro-DNA was amplified by nPCR (Table 1) targeting the gene encoding *Cryptosporidium* oocyst wall protien (COWP). Primers used to amplify 796 bp fragment were BCOWPF (5-ACC GCT TCT CAA CAA CCA TCT TGT CCT C-3) and BCOWPR (5-CGC ACC TGT TCC CAC TCA ATG TAA ACC C-3)[24]. Nested primers to amplify 553 bp fragment were Cry-15 (5-GTA GAT AAT GGA AGA GAT TGT G-3) and Cry-9 (5-GGA CTG AAA TAC AGG CAT TAT CTT G-3)[25]. The volume of reaction in the 1ry and 2ry reactions was 25 µl including 12.5 µl PCR master mix, 0.1 µl Taq polymerase, forward and reverse primer 1 µl each, 2 µl templet DNA and distilled water up to 25 µl. Reagents used in the 1ry and 2ry reactions were similar, except for the templet DNA. The amplified amplicons of 553 bp generated from the secondary reaction were subjected to 1.5% agarose gel electrophoresis and visualized by a UV trans-illuminator after being stained with ethidium bromide[24,25].

II. Genotyping: Positive samples by nPCR were then subjected to RFLP with restriction enzyme cleavage Rsa I to determine *Cryptosporidium* genotypes. The reaction was performed in a volume of 30 µl. Included reagents were 10 µl PCR product (Target DNA), 17 µl Nuclease-free water, 2 µl Green buffer and 1 µl Rsa I Enzyme. Genotype 1 was considered if Rsa I digestion resulted in 4 bands: 34 bp, 106 bp, 125 bp and 285 bp, and genotype 2 if Rsa I digestion resulted in 3 bands: 34 bp, 106 bp and 410 bp.

III. Measurement of copro-level of TNF-α: This was measured by an ELISA method (Eagle Biosciences, KR9610, USA) using fecal samples according to the manufacturer's instructions[26].

Statistical analysis: Data were coded and entered using the statistical package SPSS version 25. Data were processed using mean, standard deviation, median, minimum, and maximum in quantitative data and using frequency (count) and relative frequency (percentage) for categorical data. Comparisons between quantitative variables were done using the non-parametric Mann-Whitney test[27]. For comparing categorical data, Chi square (X^2) test was performed. Fisher exact test was used instead when the expected frequency was less than five[28]. Standard diagnostic indices including sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and diagnostic efficacy were calculated as described elsewhere[29]. Statistical significance was considered when *P* value was lower than 0.05.

Ethical consideration: Participants in this study were informed of the aim of this study and a consent was taken from each. The study followed the Ethical Guidelines of Faculty of Medicine; Cairo University Institutional which agreed with the 1964 Helsinki Declaration. *Cryptosporidium* spp. infected cases were informed and treated.

RESULTS

The stool samples were subjected to microscopic examination after permanent AF stain, ICT, ELISA and nPCR (Figure 1). *Cryptosporidium* was detected in 10 (3.7%), 17 (6.3%), 18 (6.7%), 10 (3.7%) samples, respectively. Accuracy and performance of AF stain, ICT, ELISA were calculated compared to nPCR as a reference method (Table 2). Standard diagnostic indices including sensitivity, specificity, PPV, NPV and diagnostic efficacy were calculated for each technique as described in table (3).

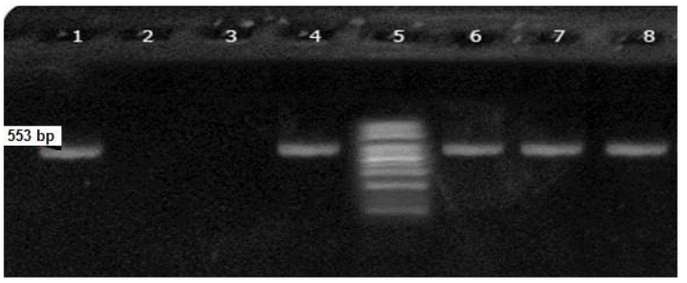

Fig. 1. Electrophoretic profiles of the PCR products amplified with the oligonucleotide primers *cowp* gene using nPCR for specific detection of *Cryptosporidium*.
Lane 5: Ready-load 100 bp ladder.
Lanes 1, 4, 6-8: Positive samples (553 bp).
Lanes 2, 3: Negative samples.

Table 2. Accuracy of AF stain, ICT and ELISA compared to nPCR.

Diagnostic method		nPCR		Statistical analysis
		Positive [No. (%)]	Negative [No. (%)]	*P* value
Microscopy	**Positive**	9 (90)	1 (0.4)	**< 0.001***
	Negative	1 (10)	259 (99.6)	
ICT	**Positive**	10 (100)	7 (2.7)	**< 0.001***
	Negative	0 (0)	253 (97.3)	
ELISA	**Positive**	10 (100)	8 (3.1)	**< 0.001***
	Negative	0 (0)	252 (96.9)	

***:** Significant (*P*<0.001)

Table 3. Standard diagnostic indices for each test.

Parameter	Microscopy		ICT		ELISA	
	Value	95% CI	Value	95% CI	Value	95% CI
Sensitivity	90.00%	55.50% to 99.75%	100.00%	69.15% to 100.00%	100.00%	69.15% to 100.00%
Specificity	99.62%	97.88% to 99.99%	97.31%	94.53% to 98.91%	96.92%	94.03% to 98.66%
PPV	90.00%	55.73% to 98.47%	58.82%	40.76% to 74.79%	55.56%	38.72% to 71.21%
NPV	99.62%	97.58% to 99.94%	100.00%	----	100.00%	----
Accuracy	99.26%	97.35% to 99.91%	97.41%	94.73% to 98.95%	97.04%	94.25% to 98.71%

ICT: Immunochromatographic test; **ELISA:** Enzyme-linked immunosorbent assay; **CI:** Confidence interval; **PPV:** Positive predictive value; **NPV:** Negative predictive value.

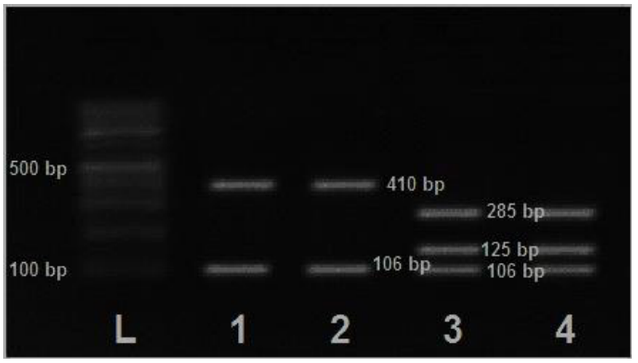

Fig 2. An agarose gel electrophoresis showing RFLP products after digestion with RsaI endonuclease.
L: DNA marker ladder.
Lanes 1, 2: *C. parvum* genotype 2.
Lanes 3, 4: *C. parvum* genotype 1.
Band 34 was too small to be detected.

To detect *Cryptosporidium* genotype, RFLP was done on nPCR positive samples. Eight samples (80%) proved to be genotype 1, and 2 samples (20%) proved to be genotype 2 (Figure 2).

Cryptosporidium was detected by nPCR in both sexes including 6 (60%) males and 4 (40%) females without any significant statistical association between sex of the patient and infection with *Cryptosporidium*. Regarding patients' residence, 7 (70%) of nPCR positive samples were related to patients living in rural areas while 3 (30%) were related to patients from urban areas with no significant statistical association. The type of drinking water among *Cryptosporidium* nPCR positive patients was tap water in 5 (50%) samples and ground water in 5 (50%) samples with significant statistical association (*P*=0.02). Level of education was also recorded and patients varied from

Table 4. Demographic and clinical data of the study population and their associated statistical significance.

Demographic and clinical data		nPCR Positive [No. (%)]	nPCR Negative [No. (%)]	Statistical analysis *P* value
Gender	**Male**	6 (60)	126 (48.5)	0.533
	Female	4 (40)	134 (51.5)	
Residence	**Rural**	7 (70)	126 (48.5)	0.212
	Urban	3 (30)	134 (51.5)	
Drinking water	**Tape**	5 (50)	216 (83.1)	**0.02***
	Ground	5 (50)	44 (16.9)	
Education	**High**	1 (10)	215 (82.7)	**< 0.001***
	Intermediate	4 (40)	43 (16.5)	
	Low	3 (30)	1 (0.4)	
	Illiterate	2 (20)	1 (0.4)	
DM	**Positive**	6 (60)	221 (85.0)	0.057
	Negative	4 (40)	39 (15.0)	
Hepatitis C	**Positive**	2 (20)	219 (84.2)	**< 0.001***
	Negative	8 (80)	41 (15.8)	
Corticosteroid therapy	**Positive**	7 (70)	125 (48.1)	0.209
	Negative	3 (30)	135 (51.9)	
Abdominal pain	**Positive**	10 (100)	223 (85.8)	0.367
	Negative	0 (0)	37 (14.2)	
Vomiting	**Positive**	5 (50)	216 (83.1)	**0.02***
	Negative	5 (50)	44 (16.9)	

nPCR: Nested polymerase chain reaction; **DM:** Diabetes mellitus, ***:** Significant (*P*<0.05).

high, intermediate, low to illiterate with significant statistical association between level of education and cryptosporidiosis (P<0.001). Furthermore, we found a significant statistical association (P<0.001) between *Cryptosporidium* and patients with positive history for hepatitis C; but we found no statistical significance with either diabetes mellitus or steroid treatment (Table 4). Regarding clinical manifestations, 100% and 50% of patients with cryptosporidiosis presented with abdominal pain and vomiting respectively, being statistically significant (P=0.02) with the latter (Table 4).

By examining copro-level of TNF-α using ELISA, it was observed that a significant statistical association existed between TNF-α and infection with *Cryptosporidium* (P=0.02) (Table 5).

Table 5. Results of TNF-α in positive and negative cryptosporidiosis by nPCR.

	nPCR		Statistical analysis *P* value
	Positive	**Negative**	
TNF-α (pg/ml) Mean ± SD	79.00±53.22	39.23±43.39	***P* = 0.02***

nPCR: Nested polymerase chain reaction; **TNF-α:** Tumor necrosis factor-alpha; ***:** Significant (P= 0.020)

DISCUSSION

The elderly are a high risk population for enteric infections due to the changes that occur in the gastrointestinal functions and the immune system with ageing[8]. Persons >65 years of age recorded a high risk of cryptosporidiosis with a shorter incubation period than reported in adults with an increased threat for secondary person-to-person transmission[30]. Mor *et al*.[31] observed a positive tendency for cryptosporidiosis among persons aged ≥65 years. The reports indicated that *Cryptosporidium* constitutes the real incidence of waterborne illness, and the association may rise with an ageing population that is principally vulnerable[31,32].

In the present study, RFLP identified 80% of the samples as genotype 1, and 20% samples as genotype 2. Similarly, Abdelrazek *et al*.[21] found that genotype 1 was more common infecting 88.3% of cryptosporidiosis patient groups, while genotype 2 infected 11.7%, with no mixed infection. It was reported that genotype 1 is restricted to humans, while genotype 2 infected different animals[24]. In contrast, a study on sporadic cases of a small outbreak in the United Kingdom illustrated that genotype 2 isolates were more frequent[33].

Regarding the association of cryptosporidiosis and diarrhea with copro-level of TNF-α, our results showed a significant statistical association with cryptosporidiosis (P=0.02). Robinson *et al*.[19] observed that porcine cryptosporidiosis was linked with histologic evidence of inflammation in the lamina propria assuming that TNF-α has a role in the pathogenesis of cryptosporidiosis. They noticed increased expression of fecal TNF-α post-*C. parvum* challenge indicating the possibility that TNF-α may synergize some undetermined factors to promote diarrhea in cryptosporidiosis. However, because there was no evident correlation between expression of TNF-α and enteric symptoms, they doubted that diarrhea in human cryptosporidiosis was principally mediated by TNF-α. Previously, Kandil *et al*.[18] suggested that TNF-α was the main mediator of diarrhea in porcine cryptosporidiosis. Seydel *et al*.[34] added that TNF-α mRNA and protein were also detected in *Cryptosporidium* infected human intestinal xenografts in immunodeficient mice. In contrast, Alcantara *et al*.[35] reported that the level of TNF-α was elevated in only one of 14 volunteers and in none of the children with cryptosporidiosis. They explained that this divergence between the stool and intestinal tissue cytokine levels was due to the greater sensitivity of tissue assays and the importance of repeating the assays to identify fecal cytokines. Moreover, proteases and bacteria in the stool samples could have damaged secreted cytokines, leading to falsely low levels.

In the present study, in comparison to nPCR, we found that out of 270 elderly patients, 10 (3.7%) were positive for *Cryptosporidium* spp. infection using the AF staining. These results were in accordance with previous results by El-Shazly *et al*.[23] who detected 5.3% positivity of cryptosporidiosis in stool samples using Z/N stain. In addition, Abd El-Kader *et al*.[36] recorded a rate of 4.6% from ten public hospitals in Cairo Governorate. Also, El-Badry *et al*.[37] reported a prevalence of 7.4% in Egyptian diarrheic children attending Cairo University hospitals. However, the present results were higher than reported by Yilmaz *et al*.[38] who found that out of 2000 children, only 1.95% were positive using microscopy of stained smears. In contrast, our results were lower than Al-Shamiri *et al*.[39] who recorded 34.7% positivity of stained smears.

In the present study, the sensitivity, specificity, and accuracy of microscopy were 90%, 99.62%, and 99.26%, respectively. A previous study by El-Hamshary *et al*.[40] certified that the modified Zeihl Neelsen stain provided a lower sensitivity and specificity of 55.3% and 79%. On the other hand, El-Missiry *et al*.[41] recorded 100% sensitivity, specificity, and accuracy of the stain. It is worth mentioning that one sample was positive by microscopy but negative with nPCR. This may be attributed to drawbacks in DNA extraction procedure, such as inefficient nucleic acid isolation or purification.

Our study showed that on performing the ICT rapid test and ELISA technique to detect *Cryptosporidium* copro-antigen, 17 (6.3%) and 18 (6.7%) were positive, respectively. These results were consistent with El-Shazly *et al*.[23] and Tahira *et al*.[42] who reported the rate of cryptosporidiosis in stool samples using ELISA as 8.3% and 11.6%, respectively. Moreover, El-Helalya *et al*.[43] and Helmy *et al*.[44] recorded 13.6% and 15.3% respective positivity of samples by ICT. By ELISA, our results were higher than those of Yilmaz *et al*.[38] who reported only 4.9% positive; and by ICT they were distinctly lower than in El-Hamshary *et al*.[40] who recorded 89.5% positive cases. Rapid-Quick ICT strip (R-Biopharm, Germany) was employed and the investigators attributed their high positivity to usefulness of the manufactured ICT. Similar results were obtained in the study conducted by Weitzel *et al*.[45] who reported that the sensitivities of RIDA-Quick, RIDA-Screen, RIDA-Quick Combi and Strip for *Cryptosporidium* were 88%, 82%, 82% and 75%, respectively.

In the present study, the sensitivity, specificity, and accuracy of the ICT (100%, 97.31%, and 97.41%) and ELISA (100%, 96.92% and 97.04%), respectively were distinctly close indicating their reliability. In another study, Zaglool *et al*.[46] reported that ICT sensitivity was 86.7% and specificity was 100%, when compared to staining. Controversially, El-Missiry *et al*.[41] recorded that ICT was 65% sensitive, 48% specific, with 50% accuracy inpatients of different ages: 214 patients ≤ 5 years, 136 ≥ 5 years; of both sexes, and different immune status. Moreover, El-Settawy and Fathy[47] recorded that ELISA was sensitive (85.7%) and specific (100%) with 96.5% accuracy in diagnosis of cryptosporidiosis compared with nPCR. Our results showed that out of the 17 samples (6.3%) positive by the ICT rapid test and 18 samples (6.7%) by the ELISA test, only 10 (3.7%) were regarded as true positive, i.e. positive by nPCR. On the other hand, 7 samples (2.6%) positive by ICT rapid test and 8 samples (2.9%) by the ELISA test were considered as false positive since they were negative by both MZN and nPCR techniques. The false positivity may be due to several reasons such as re-infections, mixed infections, the existence of antigens for several days after treatment, and the cross reactivity with other antigens[44,48].

In the present study, we found that 10 cases (3.7%) were positive using nPCR, which we considered as the reference method. Previous studies recorded 100% sensitivity and specificity in diagnosing *Cryptosporidium* spp. by PCR techniques[20,21]. Compared with our results, higher detection rate was documented by El-Hamshary *et al*.[40] who reported that multiplex PCR succeeded to diagnose cryptosporidiosis in 25% among diarrheic Egyptian children. Also, Salyer *et al*.[49] reported a higher rate (32.4%) using nPCR.

Cryptosporidiosis recorded in our study was relatively higher in males 6 (60%) than females 4 (40%) without significant association. These results were similarly cited by Qaraman *et al*.[50] who found that the infection was frequent among males than females. In contrast, Tombang *et al*.[51] found that *Cryptosporidium* spp. rate of infection was slightly greater in females 6 (5.36%) than in males 4 (3.57%). Regarding patients' residences, we noticed that the frequency of infection was more in rural areas than urban areas. Similar results were reported by Qaraman *et al*.[50]. Residence in the rural areas seems to be a contributing factor to the intensity of cryptosporidiosis risk due to poor hygiene, overcrowding, low socioeconomic conditions, and possibly contaminated water source[52]. In the present study, we found that the infection was equal among patients consuming tap and groundwater. Other studies, verified groundwater as the main source of the infection[53]. Xiao and Feng[54] reported that drinking contaminated water is an essential risk factor in the occurrence of cryptosporidiosis that was attributed to the resistance of the infecting stages to chlorine disinfection.

As regards the levels of education, the infection rate showed significant statistical association (P<0.001) with the intermediate group. Al-Shamiri *et al*.[39] recorded a high percentage in patients with illiterate parents and a low percentage with educated parents and confirmed the significant association (P=0.001) between positivity and the level of parents' education. On the other hand, Khan *et al*.[55] recorded no significant association among the patients' level of education (P=0.08).

In our work, we found a significant statistical association (P<0.001) between cryptosporidiosis and patients positive for hepatitis C. Previous studies explained that severe liver injury and liver failure are intimately associated with lowered cellular immunity[56]. In verification, Mousa *et al*.[57] reported that 32% of patients with diarrhea and hepatocellular carcinoma were infected with *Cryptosporidium*. However, we found no statistical significance between cryptosporidiosis and patients known to be diabetic, which was documented in similar studies[58,59]. Drawany *et al*.[59] explained that patients with DM might be at a higher risk for cryptosporidiosis as the clearance of parasites from the intestine might be impaired due to weakening of both innate and acquired immunities. In addition, we reported no statistical significance between cryptosporidiosis and patients under steroid treatment. In contrast, in another study a higher frequency of cryptosporidiosis *parvum* was reported in patients taking immunosuppressant medication[60].

In the present study, 100% and 50% of patients with cryptosporidiosis presented with abdominal pain and vomiting respectively, being statistically significant with the latter. In this regard, Mirzaei[61] reported that 25.6% of cryptosporidiosis cases

had diarrhea; and according to Sajjad *et al*.[62], patients included in their study had statistically significant diarrhea, abdominal pain, and vomiting. Cryptosporidiosis is a well-recognized reason for diarrhea, volume depletion, and dehydration. It was found that more than two-thirds of the reports that diagnosed cryptosporidiosis recorded volume depletion and that elderly persons may be especially prone to hospitalization due to this complication[31]. Though the asymptomatic infection was also relatively common, nonspecific low-grade fever, malaise, nausea, vomiting, and abdominal discomfort may accompany diarrhea. In chronic cases, these symptoms can lead to dehydration, weight loss, and malnutrition[63].

In conclusion, routine follow up of elderly should include stool screening for *Cryptosporidium* spp. They are considered one of the vulnerable groups due to their weak immunity rendering them more susceptible for opportunistic infections including *Cryptosporidium* spp. Although cryptosporidiosis significantly affects TNF-α in stool, further studies with larger sample size are recommended to confirm or deny this correlation.

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