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RESEARCH ARTICLE

Cryptosporidium species in HIV patients in Alexandria, Egypt: distribution and associated clinical findings

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ABSTRACT

Cryptosporidium sp. cause opportunistic infections in HIV patients. Molecular differentiation provides a better understanding of the epidemiology and clinical variations of cryptosporidiosis. The present work studied the species of *Cryptosporidium* in HIV patients and their associated demographic and clinical features. The study included 100 adult HIV patients receiving antiretroviral therapy in Egypt. *Cryptosporidium* infection was diagnosed by modified Ziehl-Neelsen (MZN) stain and PCR amplification of COWP gene. The infecting species were molecularly identified by restriction fragment length polymorphism (RFLP) and DNA sequencing. Data were analyzed using Kappa (*K*) agreement, Mann–Whitney U, odds ratio and the 95% confidence interval, Chi-squared and Monte Carlo significance (^{MC}p) tests. The statistical significance was judged at the 5% level. A total of 16 *Cryptosporidium* positive cases were detected (16%), with good agreement between PCR and MZN (*K* = 0.763). Among 11 PCR positive samples, RFLP identified *C. hominis* in five samples, *C. parvum* in three samples, *C. meleagridis* in two samples, and mixed *C. hominis* and *C. meleagridis* in one sample. Eight samples were successfully sequenced and the results confirmed the RFLP classification. *C. hominis* was found mainly in urban residents while *C. parvum* and *C. meleagridis* were significantly associated with rural areas (^{MC}p =0.01). Diarrhoea and nausea/vomiting were recorded only in the presence of *C. hominis* infection while abdominal pain was the main symptom in *C. parvum* and *C. meleagridis* infections. Drinking water sources, contact with animals, and $CD4^+$ count were not related to infection with a particular species. In conclusion, infection with *Cryptosporidium* sp. is common and frequently symptomatic in HIV patients in Egypt. The predominant species, *C. hominis,* C. *parvum,* and *C. meleagridis* show a distinct distribution in urban and rural residents.

Keywords: *Cryptosporidium*; HIV; $CD4^+$; RFLP; sequencing.

INTRODUCTION

Cryptosporidium sp. is a parasitic intestinal protozoan belonging to phylum Apicomplexa that infects a wide range of hosts including humans and animal (Ryan & Xiao, 2014; Feng *et al*., 2018). Worldwide, it is one of the leading causes of diarrhoea (Troeger *et al*., 2017). In immunocompetent humans, *Cryptosporidium* infection is usually self-limiting. However, in patients with compromised immune systems, *Cryptosporidium* sp. can be the cause of chronic diarrhoea, cachexia, lack of appetite, fever, vomiting, malnutrition and may lead to death. Infection with *Cryptosporidium* sp. is acquired through ingestion of oocysts in contaminated food or water or by direct contact with infected persons or animals (Ryan *et al*., 2016). Massive *Cryptosporidium* food-borne and water-borne outbreaks have been reported (Siwila *et al*., 2020).

The first *Cryptosporidium* species described were *C. muris* (Tyzzer, 1910) and *C. parvum* (Tyzzer, 1912). Initially, host specificity was proposed, and new species were named based on host occurrence. However, studies demonstrated that several isolates could be transmitted between different host species. The molecular characterization tools revealed the existence of multiple species (Ryan & Xiao, 2014). Information on molecular phylogenetic data collated from different parts of the world supports the taxonomic validity of 39 species (Feng *et al*., 2018; Morris *et al*., 2019). Most species are morphologically identical but can be differentiated by molecular methods. In humans, more than 20 species have been detected with *C. parvum* and *C. hominis* accounting for more than 90% of *Cryptosporidium* infections (Ryan *et al*., 2016).

Information on the infecting species and their associated clinical symptoms and transmission routes would

provide a more in-depth understanding of the epidemiology and pathogenicity of *Cryptosporidium* infection. This would assist in identifying key factors that may be used for the prediction or prevention of further infections (Ryan *et al*., 2016; Morris *et al*., 2019).

Infection with the human immunodeficiency virus (HIV) is one of the major global public health issues. It was estimated that 38 million people were living with HIV at the end of 2019, of which over two-thirds are in the World Health Organization (WHO) African Region (WHO, 2020). In Egypt, it was estimated that about 11,000 people were living with HIV in 2016 (UNAIDS, 2018). However, the relatively low prevalence of HIV infection in Egypt is attributed to the conservative culture of the local community (Boutros & Skordis, 2010) but the accuracy of these figures is questionable. Articles addressing HIV-related morbidity in Egypt and other Arab countries are generally limited. Research involving HIV patients is usually difficult due to HIV-related stigma and discrimination in Egypt (Boutros & Skordis, 2010).

In Egypt, previous reports confirmed the widespread contamination of inanimate objects and environmental samples by oocysts of *Cryptosporidium* sp., a leading cause of opportunistic infection in HIV patients. However, little is known about the prevalence and species distribution of this parasite in Egyptian HIV patients (Hassan *et al*., 2011; Hamdy *et al*., 2019).

The present work aimed to identify *Cryptosporidium* species infecting HIV-positive patients on ART in Alexandria, Egypt. Variables that might be associated with the detected species were studied.

MATERIALS AND METHOD

Study design and study subjects

A cross-sectional study was carried out on adult HIV infected patients aged 19-65 years old. They were recruited from patients admitted to or attending the outpatient clinic of Alexandria Fever Hospital during the period between February and September 2019. This hospital is located in Alexandria, the second capital of Egypt with the maximum number of immigrant populations and visitors from several surrounding areas. (Figure 1). The minimum required sample size was calculated based on 26.7% cryptosporidiosis prevalence previously reported in HIV patients (Taherkhani *et al*., 2007) and with a 10% error. A sample of 100 HIV patients was considered adequate.

Ethical and administrative considerations

Ethical approval was obtained from the Ethical Committee of the Medical Research Institute, Alexandria University (E/C. S/N. T34 /2019). All procedures involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments. The study was approved by the Ministry of Health and the hospital authority. All procedures were explained to eligible participants and informed consent was obtained before participation in the study.

Data collection

A specially designed questionnaire sheet was completed through an interview with each HIV infected patient to cover the following items: socio-demographic data (age, gender, source of water supply, contact with animals and residence) and clinical manifestations related to *Cryptosporidium* infection (as diarrhoea, vomiting abdominal colic and fever). The last $CD4^+$ cell count estimation performed at the time of enrollment in the study was obtained from the hospital record.

Collection and examination of stool samples

Fresh stool specimens free from water and urine were collected in clean, dry disposable plastic containers labeled with the patient's name and number. The containers were sealed and transported to the Parasitology Department, Medical Research Institute, Egypt.

Stool samples were homogenized by thorough mixing immediately after delivery to the laboratory. A portion of each sample was kept at -20°C in a labeled clean test tube without preservative for DNA extraction, nested PCR analysis, and genotyping.

Microscopic examination was performed using the direct wet mount with saline and iodine and the formalin ethyl acetate concentration methods for detection of protozoan trophozoites and cysts and helminths eggs. MZN staining of

Figure 1. Egypt map showing the study location.

fecal smears obtained from the concentrated fecal samples was used to detect coccidian oocysts (Garcia, 2007).

DNA Extraction and PCR amplification

Stool samples were subjected to DNA extraction using the QIAamp®stool DNA isolation Mini Kit according to the manufacturer's instructions. DNA extracts were kept at –20°C until further testing. Amplification of COWP gene was performed using a nested PCR protocol involving two consecutive reactions. The first reaction amplified the 769bp fragment by using an external pair of primer sets — 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′. The larger fragment produced by the first reaction was used as a template for the second reaction. The second reaction contained two nested primers internal to the first primer pair and delimits a 553 bp fragment. These were nest Cry-15: 5′-GT A GAT AA T GGA AGA GAT TGT G-3′ and Cry-9: 5′-G GA C TG AAA TAC A GG C AT TAT CTT G-3′ (Spano *et al*., 1997, Pedraza-Díaz *et al*., 2001). The primers were synthesized by Fermentas (Fermentas UAB, Lithuania).

The amplification reaction consisted of 12.5 µl Red Taq master mix (Bioline, UK), 1 µl (200 nmol/l) of each forward and reverse primer, 2.5 µl of template DNA, 0.1 µl Taq polymerase (5 U/µl) (product no. EP0701; Thermo Scientific), and 7.9 µl of sterile distilled water to complete a total volume of 25 µl. Reactions were performed in a gradient thermal cycler (professional thermocycler, Biometra; Applied Biosystems, California, USA) after adjusting the thermal profile to initial denaturation at 95°C for 4 min, followed by 30 cycles of amplification consisting of denaturation at 94°C for 60 s, annealing at 65°C for 60 s, and extension at 72°C for 60 s. Final elongation was performed at 72°C for 10 min. The second-round PCR was identical to the first-round PCR except for denaturation at 94°C for 50 s, annealing at 54°C for 30 s, and extension at 72°C for 50 s. The amplified PCR products were separated by electrophoresis on 2% agarose gel and visualized under a transilluminator after staining with ethidium bromide.

Restriction-fragment length polymorphism analysis

RFLP analysis was performed according to the manufacturer's instructions by digesting 10 µl of the nested PCR product (target DNA) with 1 µl of *Rsa*I (product no. ER1121; Thermo Scientific) in 2 µl green buffer and adding 17 µl nuclease-free water to reach a final volume of 30 µl. Gentle mixing was done followed by spinning down for a few seconds and then incubation at 37°C for 5 min. The restriction fragments were separated by electrophoresis on 3.2% agarose gel stained with ethidium bromide and visualized with a UV transilluminator. The detection of four bands at 34, 106, 125, and 285 bp indicates infection by *C. hominis*, while the presence of bands at 34, 106, and 410 bp is consistent with *C. parvum* (Spano *et al*., 1997; Pedraza-Díaz *et al*., 2001). *C. meleagridis* is identified by the presence of digestion fragments at 372, 147, and 34bp (Akiyoshi *et al*., 2003).

DNA Sequencing

For confirmation of RFLP typing results, positive secondary PCR products generated in the study were sequenced in one direction by the dideoxy chain terminator method using the Bigdye terminator cycle sequencing kit (Applied Biosystem, Germany). Nucleotide sequences were determined on ABI Prism 310 Genetic Analyzer (Applied Biosystems). The resultant DNA nucleotide sequences were then subjected to BLAST analysis at NCBI.

Statistical analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) software version 20 (Chicago, IL, USA). Kappa (κ) test was used to evaluate the agreement between the detection methods. Mann-Whitney U test was used to compare $CD4^+$ cell count of infected and non-infected patients. Differences between the characteristics of *Cryptosporidium* infected and non-infected patients were tested using Pearson*'s* Chi-squared test. The odds ratio and the 95% confidence interval were calculated to determine the ratio of the odds of an event in one risk group to the odds in the non-risk group. Differences between characteristics in patients infected with different *Cryptosporidium* sp. were evaluated using Monte Carlo significance test. The significance of the obtained results was judged at the 5% level.

RESULTS

Detection of Cryptosporidium infection

Microscopic examination of stool samples revealed that 15 out of 100 HIV patients (15%) had *Cryptosporidium* infection. Coinfection with *Giardia intestinalis* was found in three patients and with *Blastocystis* in two patients. The overall rate of intestinal parasitic infection was 39% with *Blastocystis* sp. being the most prevalent organism in the examined samples (22%) (Table 1).

Table 2 shows the agreement between the MZN staining method and the nested PCR assay in the diagnosis of *Cryptosporidium* infection. Ten samples showed concordant positive results, five MZN positive samples were PCR negative and one case was positive by PCR only. A total of 16 *Cryptosporidium* positive cases were detected. Statistical analysis showed a Kappa index of 0.736 (P<0.001) indicating good agreement between both techniques.

Table 1. Parasitic infection among the examined HIV patients as detected by microscopic methods

Parasitic infection	Number	%
Overall infection	39	39.0
Blastocystis spp.	22	22.0
Giardia intestinalis	4	4.0
Entamoeba coli	2	2.0
Ascaris lumbricoides	1	1.0
Cryptosporidium spp.	15	15.0
Single *Cryptosporidium*	10	10
Co-infection with *Giardia intestinalis*	3	3.0
Co-infection with *Blastocystis* spp.	2	2.0

Total number examined = 100.

Table 2. Agreement between MZN stain and nested PCR in diagnosis of *Cryptosporidium* infection among HIV patients

Nested PCR	MZN		Total
	Negative	Positive	
Negative	84	5	89
Positive	1	10	11
Total	85	15	100

Kappa index = 0.736, p <0.001*.

Molecular characterization

RFLP analysis of PCR positive samples indicated the presence of *C. hominis* in five samples, *C. parvum* in three samples, and *C. meleagridis* in two samples. One sample showed an unclear restriction pattern with probable *C. hominis* and *C. meleagridis* infection (Table 3 and Figure 2).

Out of 11 PCR positive samples, eight samples were successfully sequenced. One sample had the best match with *C. parvum* isolate with accession number (KX365886) detected in a human sample in Egypt. Another two samples showed the best match with *C.meleagridis* isolates detected in human samples in the United States and Iran with accession numbers (AY166840) and (JX568159) respectively. Of the remaining five samples, four samples showed the best match with *C.hominis* isolates isolated from humans in Egypt (KX365870, MK033078, MK033079, MK033077, MK033082) and Uganda (XM_661099). Sequencing results were consistent with the RFLP classification of the isolates.

The last successfully sequenced sample with a probable RFLP pattern of mixed infection showed several overlying peaks in the chromatogram. Upon blasting the nucleotide sequence on NCBI nucleotide blast, the sequence showed matching with both *C. meleagridis and C. hominis* suggesting mixed infection with both species. To further confirm this hypothesis, sequence pairwise alignment was performed on BioEdit (version 7.0.5.3) using the nucleotide sequence results and each of *C. meleagridis* and *C. hominis* alone. Any mismatch noticed on the alignment was checked on the chromatogram and the presence of a double peak with the expected nucleotides was confirmed (Figure 3). Two of the sequenced moleclues were submitted to GenBank and given the accession numbers MW805373 and MZ956757 for *C. meleagridis* and *C. hominis* samples respectively.

Characters associated with Cryptosporidium infection

Cryptosporidium infected patients had significantly lower $CD4^+$ cell count (median= 202.0, IQR= 143.5 – 425.0 cells/mm^3), compared to *Cryptosporidium* negative patients (median= 454.5, IQR= 377.5 – 600 cells/mm^3) (P<0.001). There was no statistically significant difference between the CD4+ counts of diarrhoeic and non-diarrhoeic *Cryptosporidium* infected patients (Table 4).

The association between *Cryptosporidium* infection and different demographic, behavioral, and clinical variables are shown in Table 5. Comparable overall *Cryptosporidium* infection rates were found among patients aged ≤40 and >40 years (15.6% and 16.6% respectively, P>0.05) as well as among males and females (16.2% and 15% respectively). Moreover, neither age nor gender showed a significant association with the identified species. The place of residence had no significant effect on the overall rate of *Cryptosporidium* infection (P=0.844). However, there was a significant association between infection by different species and patients' residence. Five out of six samples in urban areas were *C. hominis* while in rural areas, *C. parvum* and *C. meleagridis* were detected (two cases each). The patient with mixed infection was from a rural area.

Table 3. Species of *Cryptosporidium* identified in HIV patients using RFLP of the COWP gene

Cryptosporidium species	No.	%
C. hominis	5	45.4
C. parvum	3	27.3
C. meleagridis	2	18.2
C. hominis + C. meleagridis	1	9.1
Total	11	100

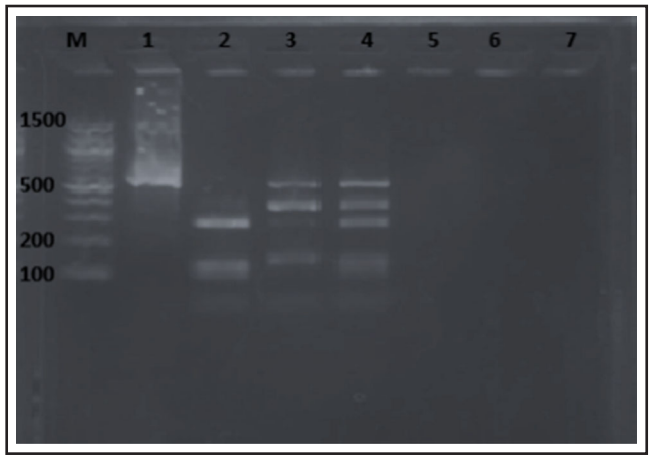

Figure 2. Agarose gel electrophoresis for the products of digestion of the *Cryptosporidium* oocyst wall protein (COWP) using RsaI. Lane (M): DNA marker ladder with numbers 100, 200, 500 and 1500 showing the bands size in bp. Lane 1: product of nested PCR before digestion at 553bp. Lane 2 showing fragments of 284,130,100 and 34 bp corresponding to *C. hominis.* Lane 3 showing fragments of 372,147 and 34 bp corresponding to *C. meleagridis*. Lane 4 shows both digestion fragments suggesting mixed infection. NB: Lanes 3,4 show also the original band at 553bp (incomplete digestion).

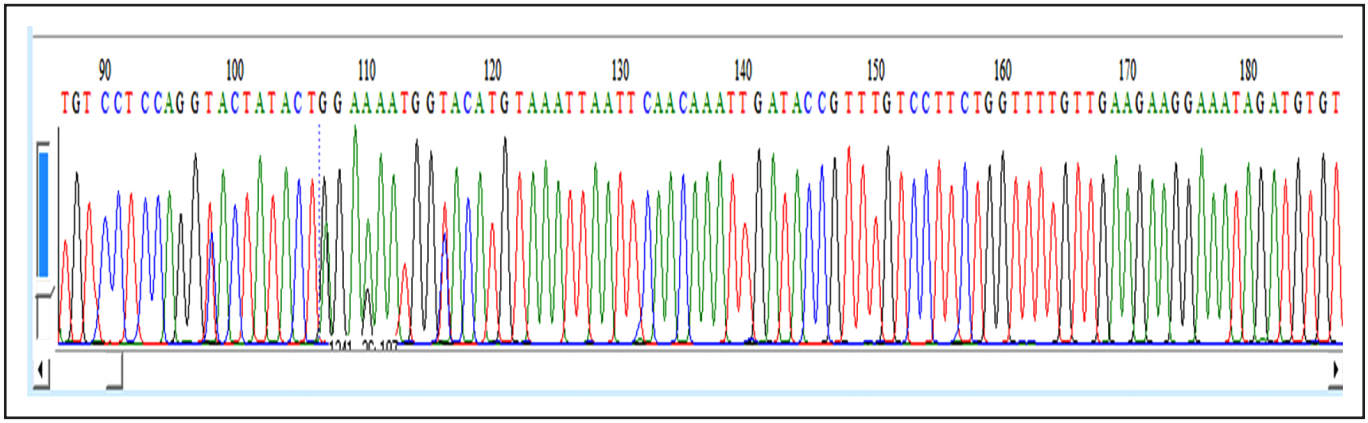

Figure 3. Double peaks in the chromatogram of sample number 16.

Table 4. CD4+ cell count (cells/mm3) in *Cryptosporidium* infected and non-infected HIV patients

CD4+ count (cells/mm3)	*Cryptosporidium* infection			
	Negative (n = 84)	Total positive (n = 16)	With diarrhoea (n=9)	Without diarrhoea (n=7)
Median	454.50	202.0	162	345
IQR	377.5 – 600	143.5 – 425.0	149 – 300	143 – 425
U	295.0*		29.0	
P	<0.001*		0.791	

Table 5. Distribution of the overall *Cryptosporidium* infection and the species identified in HIV patients on ART according to different variables

Characters	Total *Cryptosporidium* (n = 16)		p	PCR Positive (n=11)	*Cryptosporidium* spp.				$^{MC}p^1$
	No.	%			*C. hominis* (n=5)	*C. parvum* (n=3)	*C. meleagridis* (n=2)	Mixed (n=1)	
Age in years									
<40	10	15.6	0.89	7	3	1	2	1	0.81
≥40	6	16.6		4	2	2	0	0	
Sex									
Male®	13	16.2	0.89	9	5	1	2	1	0.17
Female	3	15.0		2	0	2	0	0	
Residence									
Urban®	9	16.6	0.84	6	5	1	0	0	0.01*
Rural	7	15.2		5	0	2	2	1	
Drinking water									
Tap	9	16.6	0.84	7	3	2	2	0	1.00
Filter	7	15.9		4	2	1	0	1	
Animal contact									
Yes	1	4.1	0.07	1	0	1	0	0	0.49
No	15	19.7		10	5	2	2	1	
CD4+cell count									
≤200® [2]	8	61.5	0.001*,[3]	7	3	2	2	0	0.75
>200	8	9.1		4	2	1	0	1	
Asymptomatic	2	12.5		2	1	1	0	0	1.00
Abdominal pain	11	68.8		7	2	2	2	1	1.00
Diarrhoea	7	43.8		4	3	0	0	1	0.76
Nauseas/vomiting	2	12,5		1	1	0	0	0	1.00

* Statistically significant at $P < 0.05$.
[1]: Monte Carlo, [2] Reference group,[3] Odds ratio=15.80, 95% confidence interval: 4.2 - 59.9.

Analysis of factors related to *Cryptosporidium* transmission showed that the source of drinking water and animal contact had no statistically significant association neither with the overall infection nor with the different species.

HIV patients with CD4+ counts less than or equal to 200 cells/mm3 had a 15 times higher risk of infection compared to patients with higher counts (OR = 15.80, 95% CI: 4.2 – 59.9). P=<0.001). Among the 11 genotypes samples, CD4+ count above 200 cells/mm3 was found only in four patients: one with *C. parvum*, two with *C. hominis* and one with mixed *C. hominis* and *C. meleagridis* infection. Patients with low CD4+ counts had no specific predilection for infection by a certain species.

Studying the clinical manifestations of infected patients revealed that most infections were symptomatic (14 out of 16 cases). Diarrhoea was recorded only in the presence of *C. hominis* infection either singly (3 out of 5 patients) or mixed with *C. meleagridis* (one patient). Also, nausea/vomiting was present in only one *C. hominis* infected patient. Abdominal pain was a common symptom, being recorded in two out of five *C. hominis* infected patients, and the patient with mixed infection. It was the only symptom in *C. parvum* and *C. meleagridis* single infection.

Concomitant infection with *G. intestinalis* was detected in one asymptomatic and one diarrheic patient with *C. hominis* infection as well as in the patient with mixed infection (data not shown).

DISCUSSION

In the present study, *Cryptosporidium* infection was diagnosed by microscopic methods in 15% of stool samples collected from HIV patients. In an earlier study in upper Egypt, cryptosporidiosis was diagnosed in six out of ten HIV patients complaining of diarrhoea (Dyab *et al*., 2018). Low

Cryptosporidium prevalence (3%) was previously reported among adult non-HIV patients complaining of diarrhoea in Cairo (Abd El Kader *et al*., 2012). The high prevalence of *Cryptosporidium* infection in HIV patients is related to immune suppression which increases the risk of acquiring infection from infected contacts and causes prolonged excretion of oocysts (Dyab *et al*., 2018).

In India, Ramana *et al*. (2010) reported a prevalence of 17.8% among HIV-infected patients (Ramana *et al*., 2010), whereas rates ranging from 7–9% were reported in Iran (Ghafari *et al*., 2018) and Zambia (Sinyangwe *et al*., 2020). A lower rate (2.1%) was reported in Uganda (Nakibirango *et al*., 2019). Different epidemiological conditions affect *Cryptosporidium* prevalence in HIV patients including the prevailing local living and hygienic conditions and the source of infection. Furthermore, the availability of medical care and awareness of the patients in adopting preventive measures have a significant role in limiting the spread of infection (Sinyangwe *et al*., 2020).

Previous studies reported that cryptosporidiosis particularly affects the lower age group (Khan *et al*., 2019; Elsawey *et al*., 2020). However, the present result showed that the overall *Cryptosporidium* infection, as well as the distribution of the detected species, was not significantly associated with the participants' age. This could be attributed to impairment of immune functions in all participants which renders all them vulnerable to infection regardless of their age (Rossit *et al*., 2009).

Likewise, no significant gender differences were observed. In an earlier study, it was reported that there was no significant difference in *Cryptosporidium* infection rate among males and females in any age group (Gabr *et al*., 2019). Exposure in males could be attributed to performing outdoor activities with greater exposure to unprotected food. Females could get *Cryptosporidium* infection through contact with infected children and exposure to contaminated soil in rural areas (Sinyangwe *et al*., 2020).

In the present study, there was a good agreement between MZN stain and nested PCR for the diagnosis of *Cryptosporidium* infection in HIV patients. False-negative PCR results were found in five cases whereas one PCR positive sample was MZN negative. Tahvildar and Salhi (2014) reported a low sensitivity of MZN which was attributed to the examination of stool smears without prior centrifugation (Tahvildar-Biderouni & Salehi, 2014). In the present work, using a concentration method before staining and the good experience in identifying acid-fast oocysts contributed to the good performance of MZN. On the other hand, the PCR technique for detection of *Cryptosporidium* infection is confronted by several factors. The genetic material of this protozoan parasite is enclosed within oocysts which possess a very robust cell wall resisting lysis and disruption (Surl *et al*., 2011). The presence of some fecal constituents such as hemoglobin degradation products, bilirubin, bile salts, and carbohydrates may interfere with PCR amplification leading to false-negative results (Schrader *et al*., 2012).

Molecular characterization of *Cryptosporidium* species helps in identifying the source of infection and plausible risk factors. In the present study, RFLP analysis and DNA sequencing revealed the presence of three different species of *Cryptosporidium* in HIV patients, with the anthroponotic species*, C. hominis* being the most common (45.4%) followed by *C. parvum* (27.3 %) and *C. meleagridis* (18.2%). A mixed infection (*C. hominis* and *C. meleagridi*s) was detected in one patient. In Egypt, the species of *Cryptosporidium* infecting HIV patients were not previously studied. The predominance of *C. hominis* (60%) over *C. parvum* (20%) was reported among 15 non-HIV patients in Great Cairo with some patients (20%) showing mixed infection by both species (Abd El Kader *et al*., 2012). In another study in Upper Egypt, the species identified in 112 *Cryptosporidium* infected patients were *C. hominis* (65.2%), *C. parvum* (22.3%), and *C. meleagridis* (12.5%) (Abd El Kader *et al*., 2012). *C. hominis* was more prevalent among cases of urban areas (Gabr *et al*., 2019).

Worldwide, the relative distribution of *Cryptosporidium* species in HIV patients shows some variation. In India, Dey *et al*. (2016) found that *C. hominis* and *C. parvum* genotypes were the only two *Cryptosporidium* species detected in HIV patients (Dey *et al*., 2016). In studies on HIV-infected patients from Africa (Sarfati *et al*., 2006), North America (Gatei *et al*., 2008), South America (Cama *et al*., 2003), and Europe (Llorente *et al*., 2007), *C. hominis* was the predominant species. However, in a few other studies, *C. parvum* was found to be more widespread relative to *C. hominis* (Meamar *et al*., 2006; Iqbal *et al*., 2012).

It was suggested that AIDS patients are more commonly infected with species other than *C. hominis* and *C. parvum* compared to immunocompetent individuals. *C. parvum*, *C. meleagridis*, and *C. felis* were equally detected among six HIV-positive individuals in the United Kingdom (Pedraza-Díaz *et al*., 2001). In Thailand, *C. hominis* was more common (42 cases) than *meleagridis* (20 cases) and *C. parvum* (5 cases) but other less common species were also identified namely, *C. canis* (12 cases), *C. felis* (7 cases), and *C. suis* (n = 6) (Sannella *et al*., 2019). This suggests that different risk factors may be involved in *Cryptosporidium* dissemination among HIV patients. Immunodeficiency may increase susceptibility to *Cryptosporidium* species that are not common in humans (Pedraza-Díaz *et al*., 2001).

Cryptosporidium infection can spread through contaminated water (Usluca & Aksoy, 2011). The present study showed that the source of drinking water, whether tap or filtered, had no effect on *Cryptosporidium* infection rate among HIV patients. Moreover, the three identified species were found in patients drinking tap or filtered water. This confirms the key role of water treatment facilities in providing safe tap water for human consumption with efficient removal of all species by the conventional water treatment processes.

The overall rates of *Cryptosporidium* infection among participants residing in urban and rural areas were nearly similar. However, *C. parvum* and *C. meleagridis* infections were significantly associated with rural areas while *C. hominis* was found mainly in urban residents who were not dealing with animals. Animal contact was recorded in one patient who was infected with *C. parvum.* Previous studies confirmed that most cases infected with *C. parvum* and *C. meleagridis* species lived and bred animals in rural areas (Gabr *et al*., 2019; Sannella *et al*., 2019). Lack of hygiene, poor living conditions, and direct contact with farm animals enhance the spread of infection (Usluca & Aksoy, 2011; Sinyangwe *et al*., 2020). *C. meleagridis* has been isolated from birds and humans with relatively low host specificity (Cama *et al*., 2003; Liao *et al*., 2018). It is well documented that most *C. parvum* strains are zoonotic while few are almost entirely anthroponotic and predominate in areas with poor sanitation and in HIV patients (King *et al*., 2019). The association between the infecting species and the patient's residence in the present study indicates that animals act as a potential source of human infection in rural areas. This should be confirmed by further local studies involving the characterization of isolates from infected animals. The non-significant relation between animal contact and the infecting species points to the possible occurrence of indirect or anthroponotic transmission of some zoonotic species after

its introduction into the human host. Noteworthy, the information on animal contact in the present study was based on the patients' response to the questionnaire from and casual contact with animals cannot be correctly ruled out in all cases.

The current study showed that patients with low CD4+ cell count were at higher risk of cryptosporidiosis with equal susceptibility to the three identified species. In HIV/AIDS patients on ART, all species of *Cryptosporidium* are known to resolve spontaneously with immune restoration (Alfonso & Monzote, 2011). It has been well documented that cryptosporidiosis occurs mainly in patients with CD4+ counts below 200 cells/mm^3 and that patients with a CD4+ count below 50 cells/mm^3 are at high risk of severe infections (Kulkarni *et al*., 2009; Mohebali *et al*., 2020). However, the present study showed a non-significant difference between diarrhoeic and non-diarrhoeic *Cryptosporidium* infected patients regarding CD4+count. This may be explained by the occurrence of diarrhoea in some infected patients as a result of other factors such as ART or associated bacterial and viral infection (Dikman *et al*., 2015).

In the current study, most *Cryptosporidium* infected patients had one or more gastrointestinal related symptoms. Diarrhoea and nausea/vomiting were recorded only in the presence of *C. hominis* infection while abdominal pain was the main symptom in patients with single *C. parvum* or *C. meleagridis* infection. Thus, infections caused by *C. hominis* seem to induce more severe infection although the contribution of associated *G. intestinalis* to gastrointestinal symptoms in some patients cannot be excluded. Previous studies showed that infection by *C. hominis* is associated with greater parasite load and more frequent diarrhoea, nausea and vomiting compared to *C. parvum* (Cama *et al*., 2008; Dey *et al*., 2016; Elsawey *et al*., 2020).

Among the studied patients, *Cryptosporidium* sp. was the second most common parasites, being next to *Blastocystis* sp. (22%), a protozoan with a controversial pathogenic role (Andersen & Stensvold, 2016). The non-opportunistic parasite, *G. intestinalis* was detected at a relatively lower rate (4%). In HIV patients, the rate of infection with non-opportunistic parasites depends on their endemicity in the community (Rao, 2016). The high prevalence rate of *Blastocystis* colonization and the lower rate of giardiasis are in agreement with reports of previous surveys conducted on populations of a similar age range in Egypt (Banisch *et al*., 2015; Dyab *et al*., 2018).

In conclusion, infection by *Cryptosporidium* sp. is common and frequently symptomatic in HIV positive patients receiving ART in Egypt. Immunosuppression, as determined by low CD4+ count, was the single most important risk factor for *Cryptosporidium* infection in the studied patients with no predilection for infection by a certain species. The predominant species, *C. hominis*, *C. parvum* and *C. meleagridis* showed distinct distribution in urban and rural residents. Improved hygiene and avoidance of animal contact should be advocated to reduce *Cryptosporidium* infections among HIV patients. Further genotyping studies of *Cryptosporidium* sp. isolated from human, animal, and environmental samples are needed to identify the potential reservoirs and sources of infection.

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Conflicts of Interest

The authors declare there are no conflicts of interest.

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