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CRYPTOSPORIDIOSIS AMONG OUTPATIENTS WITH DIARRHEA IN EL FAYOUM GENERAL HOSPITAL EGYPT: DIAGNOSIS AND RISKS

By
FATEN AHMED M. MOHAMED[1]* and MOHAMED MASOUD[2]
[1]Department of Medical Parasitology and [2]Department of Public Health, Faculty of Medicine, Fayoum University, Egypt (Correspondence: fam02@fayoum.edu.eg)

Abstract

Cryptosporidium is a significant zoonotic parasite causing a self-healing gastroenteritis; with *C. hominis* and *C. parvum* are the main agents of cryptosporidiosis. This study examined stool samples from diarrheic patient live in rural areas in Fayoum Governorate, to identify causative species and risky factors associated with zoonosis. Of 152 stool samples were collected from December 2020 to February 2021 from outpatient clinics in El Fayoum General Hospital. Screened smeared by MZN stain detected oocysts in 26 samples, and Copro-ELISA assay detected positive in 32 (21.05%). By nested PCR targeting 18s rRNA gene, 11 samples were amplified. *Cryptosporidium* genotyping by REFLP showed predominance of *C. parvum* genotype followed by *C. hominis*. This was supported by highly risky association (P ≤ 0.05, OR =0.186 [0.061-0.562]) found between infected patients and animals contact.

Key words: El-Fayoum, Outpatients, Cryptosporidiosis, Diagnosis, Risk factor.

Introduction

Cryptosporidiosis is an opportunistic enteric disease caused by *Cryptosporidium* species (Ryan *et al,* 2016). *C. hominis* and *C. parvum* are the most frequent zoonotic ones (Chalmers and Davies, 2010). Parasitic oocysts transmitted through contaminated food and water so it is consider as a waterborne pathogen (Tahvildar-Biderouni and Salehi, 2014).

The parasite invades the intestinal epithelial cells of hosts causing watery diarrhea (Nahrevanian *et al,* 2010). Diarrhea is mild among immunocompetent (Tumwine *et al,* 2005) but may be profuse and fatal in immunocompromised patient. Other gastrointestinal symptoms may be abdominal pain, vomiting and fever (Chalmers and Davies, 2010).

Disease is widely distributed (Putignani and Menichella, 2010). WHO has included it as one of the neglected diseases (Cacciò and Chalmers, 2016). *Cryptosporidium* infections are endemic In developing countries (Fayer, 2004). The prevalence rate in Egypt ranged (1 to 43%) among immunocompetent individuals with diarrhea (Abo-Sheishaa, 2015).

Diagnosis depends on microscopic examination of stained smears (Ahmed and Karanis, 2018). Oocysts appear round pink with four sporozoites with blue background by NZN stain (Vanathy *et al,* 2017), and easily by antigen based ELISA (Khurana and Chaudhary, 2018).

Hamdy *et al.* (2019) reported that several genes were used for oocyst wall protein (COWP), dihydrofolate reductase (DHFR), thrombospondin-related adhesive protein 1 (TRAP- C1), thrombospondin-related adhesive protein 2 (TRAP-C2), internally transcribed spacer 1 (ITS1), polythreonine repeat (Poly-T), small-subunit (SSU) rRNA genes. The analysis of the small subunit 18S rRNA gene are used to detect the parasite DNA, and identify species and genotypes in animal, human, and environmental samples (Köseoğlu *et al,* 2022).

The current study aimed to trace cryptosporidiosis among diarrheic outpatient clinics in El Fayoum General Hospital, El Fayoum Governorate, by MZN stain and ELISA, and to identify risky factors and prevalent species.

Material and Methods

Study Design: A cross sectional study was carried out from December 2020 to February 2021. It aimed to screen 152 patients attending outpatient clinics in El Fayoum General Hospital, El Fayoum Governorate, Egypt.

The study group aged from 20 to 65 years old and presented with diarrhea, with or

without other associated symptoms as nausea, vomiting, abdominal pain, flatulence and/or fever. Patients had antiparasitic or antidiarrheal treatment before examination was excluded from the study.

Ethical consideration: The study protocol was approved by the Scientific Research Ethics Committee, Faculty of Medicine, Fayoum University, R252 on 11/9/2022. Study aims were described for each participant, and have the right to withdraw. The demographic characters and symptoms were recorded on preplanned sheet.

Stool examination: Fresh stool samples were collected from all patients in 60ml clean labeled containers. Each sample was divided into 2 parts; 1st part for staining was preserved in 10% formalin and concentrated by modified formalin ethyl acetate procedure (John and Petri, 1999), and 2nd part was stored at -20°C without preservative to detect antigen by sandwich ELISA and fecal Nested PCR-REFLP assay.

Copro-staining and microscopic examination: Air-dried smears were prepared from stool concentrate were fixed with methanol and then stained separately by cold kinyoun stain (Egyptian Dignost. Co), and examined by a research microscope to detect oocysts.

Antigen detection: Frozen portion of each specimen was tested for *Cryptosporidium* copro-antigen by RIDASCREEN® *Cryptosporidium* ELISA Kit (Biopharm, Darmstadt, Germany) after manufacturer's instructions.

Cryptosporidium genotyping by nested-PCR/Restriction fragment length polymorphism (RFLP): DNA was extracted from frozen positive samples using the QIAamp Stool Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The purified DNA was subjected to n-PCR to study Cryptosporidium isolations. 2 primers sets targeting 18s rRNA gene were used for primary and secondary nested PCR reactions. The following protocol were used: 3min initial denaturation step at 94°C, followed by 35 cycles of 45s at 94°C, 45s at 55°C, and 1min at 72°C, and then extended to 7min at 72°C. Amplification followed by RFLP analysis using SspI & VspI (Thermo-Fisher, USA) digestion after manufacturer's instruction. Restriction assay was performed in a 30µl volume with 0.5units of restriction enzymes and 20µl of PCR product/reaction, and were incubated at 37°C for 8hrs. Digested products were visualized under UV light after 2.5% agarose gel electrophoresis & ethidium bromide staining (Xiao *et al,* 1999).

Primers sets used in nested – PCR reaction.

Primer set	Sequence	Product size
SSU-F2	5´- TTCTAGAGCTAATACATGCG -3´	1325bp
SSU-R2	5´- CCCATTTCCTTCGAAACAGGA-3´	
SSU-F3	5´- GGAAGGGTTGTATTTATTAGATAAAG -3´	826–864bp
SSU-R4	5´- CTCATAAGGTGCTGAAGGAGTA -3´	

Statistical analysis: Data were analyzed using SPSS version 23. Frequency distribution for variables was done first. Mean ±SD were used for parametric numerical data. Variances and correlation between parameters and parasitic determination were checked by chi-square test and t-student test. Odds Ratio (OR) adjusted for all other variables (95% confidence intervals). In comparing the ELISA methods with acid-fast technique as a standard test, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and diagnostic accuracy were calculated. Kappa agreement (K) was used for measuring the agreement between staining results and ELISA (K < 0.2 poor agreement, K 0.2-0.4 fair agreement, K 0.41-0.6 moderate agreement, K 0.61-0.8 good, and K0.81-1.0 very good agreement). P< 0.05 was considered significant.

Results

Of 152 stool samples examined, *Cryptosporidium* oocysts were detected in 26 (17.1%) and 32 (21.05%) samples by MZN stain and ELISA respectively. Stained smears showed oocysts as 3-6µm bright red ovoid structures against blue background, with thick walls and four dark sporozoites difficulty recog-

nized in some oocysts. Of 126 samples negative by stained smears, six were positive by ELISA. Using stained smear as a golden standard, sensitivity and specificity of stool ELISA was 100% (CI: 86.77% to 100%) and 95.24% (CI: 89.92% to 98.23%), respectively, and PPV, 81.25% (CI: 66.50% to 90.44%) and NPV, 100%. Accuracy 96.05% (CI: 91.61% to 98.54%), Kappa was almost perfect agreement (0.872). Mean ages of positive cases was 38.3±2.3years (87.5%) within age group (21-60years). Also, infection was higher in ma-les (59.4%) than females (40.6%), without differences in risk of infection [OR= 0.895 (CI= 0.405-1.976)]. The risk factors didn't show significant association ($P \geq 0.05$) between cryptosporidiosis and patient's age, sex and water source. *Cryptosporidium* rate was significantly higher ($P \leq 0.05$) in patients with indoor animals.

Clinical presentation: diarrhea (71.9%), abdominal pain (65.6%), vomiting (56.3%), flatulence (43.8%), nausea (40.6%), fever (28.1%) and tenesmus (12.5%), with significant correlated ($P \leq 0.05$) followed by abdominal pain, vomiting, nausea and then fever.

A total 11/32 positive stools for DNA extraction and nested PCR amplification were successfully amplified at 830bp. *Cryptosporidium* molecular identified by RFLP on 11 nPCR positive samples showed that *C. parvum* 8 (72.7%), and *C. hominis* 3 (27.2%).

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

Table 1: Cryptosporidiosis according to participants' age and sex:

Variation		Positive	Negative	P-value
		Mean ±SD	Mean ±SD	
Age		38.3±2.3	41.9±1.2	
Age groups	<=20	1(3.1%)	9(7.5%)	0.179
	21-60	28(87.5%)	107(89.17%)	
	>61	3(9.4%)	4(3.3%)	
Sex	Male	19 (59.4%)	68 (56.7%)	0.783
	Female	13(40.6%)	52(43.3%)	
Total		32	120	152

Table 2: Risk of cryptosporidiosis and social parameters:

Variations		Positive (N=32)		Negative (N=120)		P-value	Odds Ratio
		No.	%	No.	%		
Animals indoor	No	8	25%	7	5.8%	0.001*	0.186
	Yes	24	75%	113	94.2%		(0.061-0.562)
Water	Filtered	3	9.4%	5	4.2%		0.420
	Tape	29	90.6%	115	95.8%	0.241	(0.095-1.861)

Table 3: Risk estimates of cryptosporidiosis within clinical pictures among group:

Clinical pictures		Positive (N=32)		Negative (N=120)		P-value	Odds Ratio
		No.	%	No.	%		
Abdominal pain	No	11	34.4%	80	66.7%%	0.001*	3.818
	Yes	21	65.6%	40	33.3%		(1.678-8.689)
Vomiting	No	14	43.8%	96	80%	<0.001*	5.143
	Yes	18	56.3%	24	20.0%		(2.244-11.787)
Diarrhea	No	9	28.1%	53	44.2%	0.101	2.022
	Yes	23	71.9%	67	55.8%		(0.863- 4.733)
Flatulence	No	18	56.3%	83	69.2%	0.169	1.745
	Yes	14	43.8%	37	30.8%		(0.785-3.878)
Nausea	No	19	59.4%	97	80.8%	0.011*	2.886
	Yes	13	40.6%	23	19.2%		(1.247-6.679)
Tenesmus	No	28	87.5%	101	84.2%	0.640	0.759
	Yes	4	12.5%	19	15.8%		(0.239-2.414)
Fever	No	23	71.9%	116	96.7%	<0.001*	11.348
	Yes	9	28.1%	4	3.3%		(3.219-40.002)

Odds Ratio (OR) adjusted for all other variables (95% confidence intervals), *$P < 0.05$ significant.

Discussion

In the present study, overall cryptosporidiosis was 14.6% (CI: 9.77% -20.67%) which agreed with Egyptian ranges reported (Rafiei *et al,* 2014; Youssef and Uga, 2014).

The oocyst was detected (17.1%) by acid

fast stained smear and (21.05%) by coproantigen ELISA. This agreed with El-Settawy and Fathy (2012) who detected oocyst (18.6) by microscopy and (20.9%) by coproantigen ELISA. Al-Hindi *et al.* (2007) in Gaza and El-Sayed and Méabed (2010) in Egypt reported (14.9%,16.3% & 10.2%, 15.7%) by acid fast stain and ELISA respectively. But, Abo-Sheishaa (2015) reported oocyst detection decreased (5.43%) by Z/N stain, and (9.96%) by ELISA. But, Abdelhafeez *et al.* (2012) reported a high rate of 42.2%, 60.2% in immunocompetent and immunosuppressed individuals respectively.

In the present study, using stained smear as a golden standard, ELISA sensitivity, specificity, and accuracy was (100%, 95.24% & 96.05%). It's PPV, NPV (81.25% &100%) with perfect agreement Kappa (0.872). Oocysts also might not be detectable in samples from all cases due to poor uptake of stain by oocysts, so ELISA assay were used for antigen detection (Khurana *et al,* 2012). This could explain the extra ELISA 6 positive samples.

In the present study, zoonotic *C. parvum* and *C. hominis* were (72.7% & 27.2%) detected respectively by the nested-PCR/REFLP. This indicated that zoonotic transmission is more prevalent especially with farming areas. This agreed with Iqbal *et al.* (2011) in Kuwait, Rafiei *et al.* (2014) in Iran and Eraky *et al.* (2015) in Egypt. But, Naguib *et al.* (2018), El-Missiry *et al.* (2019); Mohammad *et al.* (2021) and Amin *et al.* (2021) found *C. hominis* was the main zoonotic genotype.

In the present study, there was no significant difference between age groups; highest one (87.5%) was in 21-60. This agreed with Adler *et al.* (2017). But, Bouzid *et al.* (2018) reported that crowded living conditions, animal contact and open defecation are responsible for the *Cryptosporidium* majority cases in low and middle income countries.

In the present study, cryptosporidiosis was higher in males than females without risk association, which could be explained by nature of male activity in soil as gardens and farms and animals contact. This agreed with Abdel Gawad *et al.* (2018); Khan *et al.* (2019) and Mohammad *et al.* (2021). But, Adler *et al.* (2017) found a significantly high cryptosporidiosis risk in males than females.

In the present study, a highest rate (90.6%) was detected among tap water users without a risk factor ($P \geq 0.05$). This agreed with El-Sherbini and Abosdera (2013) and El-Missiry *et al.* (2019). But, Khan *et al.* (2019) linked between water contaminated by fecal cryptosporidiosis oocyst as a risk factor.

In the present study, there was high significantly infection rate ($P \leq 0.05$) among patients having indoor animals. This agreed with El-Sherbini and Mohammad (2006), who found positive significance ($P < 0.01$) between farmers and zoonotic infected farm animals. The risk of zoonosis in Egyptian rural villages related to livestock and pets animals without public knowledge was reported (Ahmed *et al,* 2016; Ismail *et al,* 2018). Khan *et al.* (2019) reported that contact with infected animals was an important mode of transmission. But, Madi (2019) and Moh-ammad *et al.* (2021) didn't find a correlation between infection and animal contact.

In the present study, the symptoms were diarrhea (71.9%), abdominal pain (65.6%), vomiting (56.3%), flatulence (43.8%), nausea (40.6%), fever (28.1%) and tenesmus (12.5%), with *Cryptosporidium* significant risk ($P \leq 0.05$) and abdominal pain, vomiting, nausea and fever. Sajjad *et al.* (2014) found that significant risks were abdominal pain ($P< 0.001$), vomiting ($P< 0.001$), and low grade fever ($P< 0.001$). But, El-Sayed and Méabed (2010) and Khan *et al.* (2019) reported that patients with diarrhea were at greater risk for cryptosporidiosis.

Conclusion

Cryptosporidium parvum and *C. hominis* causing diarrhea were in rural areas. Significant risky factor of animal contact was the cause of zoonotic infection. Cryptosporidiosis massive control must be considered.

Authors declared that they neither have conflict of interest nor received any funds.

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Explanation of figures

Fig. 1: *Cryptosporidium* oocysts seen as bright red ovoid structures against blue background.

Fig. 2: PCR-RFLP of *Cryptosporidium* SSUrRNA Gene: Lane 1, DNA marker size. Lane 2, undigested nested PCR product (830bp). Lanes 3-5, *C. hominis* 556 and 116 bp. Lanes 6-8 *C. parvum* 628,104 bp.