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EVALUATION OF *CRYPTOSPORIDIUM* INFECTION IN IMMUNOCOMPROMISED AND IMMUNOCOMPETENT CASES BY STOOL ANALYSIS VERSUS COPRO-ELISA

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Abstract

Cryptosporidium parvum is an opportunistic intracellular apicomplexan parasite that can cause life-threatening diarrhea in immunocompromised patients and self-limited diarrhea in healthy persons. This study screened *Cryptosporidium parvum* infection among immunocompromised patients versus immunocompetent persons, comparing different diagnostic methods of *Cryptosporidium parvum,* and to detect the validity of mini FLOTAC apparatus versus the coproantigen ELISA test. A cross-sectional study was carried on two groups: G1:100 immunocompromised patients recruited from the National Liver Institute, Oncology Institute, Renal Dialysis Unit, over four consecutive seasons. G2: 100 immunocompetent apparently healthy persons. Stool samples were divided into two parts, the first part was examined freshly by direct smear and staining methods, some mixed with formalin 10% for FECM, and some was used in mini-flotac technique mixed with zinc-sulphate solution. The second part was frozen in (-20) freezer for later ELISA copro-antigen technique. The samples were *C. parvum* oocyst examined by direct smear, staining (Iodine and/or Modified Ziehl-Neelsen) and Mini-Flotac apparatus versus *C. parvum* antigen by coproantigen ELISA as a golden standard test.

The results showed that *Cryptosporidium parvum* infection by copro-antigen ELISA test was 25% among all participants (20.5%) in immunocompromised and (4.5%) in immunocompetents respectively. Regarding diagnostic tests, direct smear had the lowest sensitivity (14%) for *Cryptosporidium Parvum* detection while the Mini-FLOTAC technique had the highest sensitivity (84%) compared to the copro-antigen ELISA method which was 100%.

Keywords: *C. parvum, S*tool analysis, Mini-FLOTAC test, Copro-antigen ELISA.

Introduction

C. parvum is one of the worldwide prevalent intestinal parasites with significant importance, particularly among immunocompromised patients can cause chronic life-threatened diarrhea and malabsorption (Piazzesi *et al,* 2023). Cryptosporidiosis most common symptoms (2-10 days or 7 days) is prolonged, frequent, and watery diarrhea, also stomach cramps, pain, nausea, vomiting, fever, weight loss, and dehydration (CDC, 2024). Symptomatic and asymptomatic Cryptosporidiosis in children was sometimes associated with malnutrition and stunted growth (Moore *et al,* 2010). Over 2.9 million of cryptosporidiosis occur every year in children aged < 24 months in Sub-Saharan Africa (Tombang *et al,* 2019). The waterborne protozoa as *Cryptosporidium* and *Giardia* species survive (33.3% & 20.8% respectively) in the River Nile despite the use of chlorine disinfectants (El-Khayat *et al,* 2022). Oocysts are transmitted by the fecal-oral route, or with contaminated raw vegetables, fruits, or water and/or contact with a patient or farm or domestic animals (El-Bahnasawy *et al*, 2018). Also, human-to-human was reported (Bouzid *et al*, 2013).

Cryptosporidiosis as other protozoa was simply diagnosed by the microscopy stained smears (El Naggar *et al,* 2006**)**. Their tiny, microscopic size, staining absorbance, and

oocysts morphology made the smears time-consuming needing an expert technician detect oocysts, which sporadic shedding added to diagnostic challenge (Ahmed *et al,* 2023).

ELISA assay to identify the *C. parvum* copro-antigens in stools was used and proved to dependable in laboratory and epidemiological studies as quick, and standard than routine stained smears diagnosis (El-Settawy and Fathy, 2012). Also, the Mini-FLOTAC proved to a safe rapid device for microscopy stool samples examination with high sensitivity, affordability, and appropriateness in di agnosing many intestinal parasites (El-Nadi *et al*, 2019).

The present study aimed to screen *Cryptosporidium parvum* infection among immunocompromised patients in the National Liver Institute, Department of Oncology and Renal Dialysis Unit, versus immunocompetent persons, and to evaluate the Mini-FLOTAC technique validity versus different microscopy stained smears considering the copro-antigen ELISA as the golden standard test.

Subjects and Methods

Ethical consideration: This study was approved by the Institutional Review Board of the National Liver Institute, Menoufia University (00608/2024). Written informed consent was given by patients and all procedures were explained to them and all the participant names were replaced by code numbers to maintain their privacy. Demographic data and information about potential risk factors for both groups were collected using a standard designed questionnaire form.

Study design: This cross-sectional study was carried out on two groups of 100 part icipants each. G1: Immunocompromised patients were recruited from the Liver Transplant Surgery Department, Oncology, Nephrology Clinics, and Renal Dialysis unit, National Liver Institute, and Oncology Institute. They were then divided into 4 subgroups of 25 each. SGa: Patients underwent liver transplantation, SG1b: Patients with liver malignancy on chemotherapy, or radiotherapy, SGc: Patients on hemodialysis drug and SGd: Patients with chronic debilitating diseases. G2: Cross matched immunocompetent apparently healthy persons.

Samples collection: A small morning stool sample was obtained from each one in a clean, labeled, wide-mouth disposable covered container, and microscopy examined using: 1- Direct smear and 2- Formalin-ether concentration method; re-centrifuged sample showed 4 layers, top ether dissolved fats and lipids, second one fecal debris, a third liquid formalin and last sedimented parasites were microscopy examined (Sharaf *et al*, 2021). 3- Iodine and modified Ziehl-Neelsen stains Methods for 2mg was taken from interior & exterior samples on slide, and stained with Logo's iodine solution1/5 buffered diluted solution. Smear was stained with MZN air dried, coated with alkaline Fuschin for 5 minutes later rinsed in water and discolored with 2.5% Sulfuric acid for a minute, counterstained with 1% methylene blue for a minute, air dry again and microscopy examined (Shalaby and Shalaby, 2015). 4- Mini-flotac is simply done, cost-effective, and suitable for field and laboratory studies with accurate results. Stool sample after mixed with a 1.35 specific gravity zinc sulfate flotation solution (FS); FS7 with a specific gravity higher than the oocysts, Mini-FLOTAC two chamber sections were loaded by homogenized mixture, and centrifuged or left undisturbed, allowing oocysts to float on upper surface and examined microscopically (El-Nadi *et al,* 2019). 5- Imm-unological diagnosis using antibody or dete- cted antigen gave high sensitivity and speci- ficity, as ELISA, Immunochromatographic tests, Immunochromatographic lateral flow (ICLF), Immunofluorescence assays (IFA) & flow cytometry coupled with cell sorting. The nanotechnology-based platforms proved to be sensitivity and specificity than ELISA, ICLF, IFA or even PCR (Aboelsoued and Abdel Megeed, 2022).

Statistical analysis: Data were collected, computerized and analyzed using Statistical Package of Social Science (SPSS) version

20 (Inc., Chicago, Illinois, USA). Association between patients' immunological status, symptoms, linked traits, and infection was assessed by using the Chi-square test. Also, Fisher exact test determined the average data. P value was significant if it was < 0.05.

Results

Of the 200 immunocompromised and immunocompetent cases, 50 (25%) were *C. parvum* positive by ELISA, and 41% in immunocompromised cases higher than 9% in immunocompetent ones (P= 0.001). Mini-Flotac test showed 23%, followed by MZN st ain 21%, and least was in direct smear 7.5%.

The sensitivity (94%) occurred with mini-flotac test, both MZN stain after FECM showed (84%) sensitivity, iodine-stained smears showed (46%) and least was the direct smear (30%). Between the subgroups of immunocompromised patients, there was significant variation *Cryptosporidium* infection prevalence by copro-antigen ELISA. Positive samples were highest in liver transplantation patients (39%) followed by chronic debilitating patients; diabetic, COPD, Rheumatic, chronic kidney (34.1%), cancer patients (17.1%) and least renal dialysis patients (9.8%) with (P= 0.001).

Positive infection rates was higher in the immunocompromised patients aged 14-40 years and 41-65yrs compared to immunocompetent persons aged >65yrs. The males (58%) showed more infected than females (42%), but without significant differences. Positive cases were more in cases from rural areas (68%) than those in urban (32%) ones.

Seasonal fluctuation of cryptosporidiosis rates increased in summer (50%), declined in autumn (24%), dropped in winter (16%), and then followed by spring (10%). Patients used tap water showed more positive rate (76%) than those on filtered water (24%).

Diarrhea was high in the immunocompromised cases (70.7%) than in immunocompetent ones (29.3%) with marked significant (P= 0.019).

Details were given in tables (1, 2, 3, 4 & 5) and figures (1 & 2)

Table 1: *Cryptosporidium* detected by different examination methods:

Diagnostic methods	Positive		Negative	
	No.	%	No.	%
Direct smear	15	7.5	185	92.5
Iodine staining	23	11.5	177	88.5
MZN staining	42	21.0	158	79.0
Mini-flotac	47	23.5	153	76.5
Coproantigen	50	25.0	150	75.0

No: number %: percentage

Table 2: Sensitivity and specificity of all diagnostic methods as compared to coproantigen ELISA

Diagnostic methods	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
Direct smear	30%	100%	100%	81.0%	82.5%
Iodine staining	46%	100%	100%	84.7%	86.5%
MZN staining	84%	100%	100%	94.9%	96.0%
Mini-flotac	94%	100%	100%	98.0%	98.5%
ELISA test	100%	100%	100%	100%	100%

PPV: Positive predictive value. NPV: Negative predictive value.

Table 3: *C. parvum* among different immunocompromised patients by copro-antigen ELISA (n=100):

Clinical variations	Positive (n =41)		Negative (n =59)		X2	P value
	No.	%	No.	%		
Liver transplantation patients (N=25)	16	39.0	9	15.3	15.9	0.001**
Cancer patients (N=25)	7	17.1	18	30.5		
Renal dialysis Patients (N=25)	4	9.80	21	35.6		
chronic debilitating disease (N=25)	14	34.1	11	18.6		

**highly significant

Table 4: Comparison between both true positive cases (ELISA) as to socio-demographic data:

Studied variables	Immunocompromised (n=41)		Immunocompetent (n=9)		Total positive		X2	P value
	No.	%	No.	%	No	%		
Age/years 15-40	29	70.7	0	0.00	29	58.0		
41- 65	12	29.3	0	0.00	12	24.0	X2	<0.001**
>65	0	0.00	9	100.0	9	18.0	50.0	
Male	24	59.0	5	55.6	29	58.0	FE	
Female	17	41.0	4	44.4	21	42.0	0.02	0.870
Residence: Rural	28	68.3	6	66.7	34	68.0	FE	
:Urban	13	31.7	3	33.3	16	32.0	0.01	0.925
Working	33	80.5	6	66.7	39	78.0	FE	
Not working	8	19.5	3	33.3	11	22.0	0.822	0.365
Spring	4	9.70	1	11.1	5	10.0		
Winter	7	17.1	1	11.1	8	16.0	X2	
Summer	18	43.9	7	77.8	25	50.0	4.50	0.212
Autumn	12	29.3	0	0.00	12	24.0		
Filter water	10	24.4	2	22.2	12	24.0	FE	
Tab water	31	75.6	7	77.8	38	76.0	0.02	0.890

P value= positive.

Table 5: Comparison between both positive *Cryptosporidium* infection by copro-antigen ELISA test as to diarrhea

Diarrhea variation	Immunocompromised +ve (41)		Immunocompetent +ve (9)		Total +ve (50)		Fisher exact	P value
	No.	%	No.	%	No.	%		
Positive	29	70.7	2	22.2	31	62.0		
Negative	12	29.3	7	77.8	19	38.0	5.45	0.019*

No: number %: percentage *significant

Discussion

In the current study, *C. parvum* infection rate by the gold standard copro-antigen ELISA was 50/200(25%) of all cases. This more or less agreed with a cross-sectional study by Elshahawy and Abouelenien (2019) in Qena Governorate, who by ELISA reported 20.83% *C. parvum* among patients. A very low cryptosporidiosis rate was reported by Liu *et al.* (2020) 2.97% (200,054) by copro-ELISA. A very high rate (87.57%) was reported by Mohammed *et al.* (2023) in Iraq. These different cryptosporidiosis rates might be due to difference in geographical countries, environmental conditions, animal reservoirs, and human cultures.

In the present copro-antigen ELISA study, the immunocompromised cases showed high *C. parvum* rate (41%) as compared to immunocompetent one (9%). This more or less agreed with Elsawey *et al*. (2020) in Egypt, who reported total cryptosporidiosis *parvum* of 59% were (84%) among 150 immunocompromised schoolchildren and (34%) in immunocompetent ones.

In the current study, immunocompromised patients showed positive cryptosporidiosis rate in liver transplantation patients followed by chronic debilitating diseased ones, and then cancer patients, and the least was in renal dialysis patients. This more or less agreed with Ahmed *et al*. (2023) in Egypt, who reported *C. parvum* 15% in liver transplant recipients (OTRs). But, this disagreed with Al-Shehari *et al*. (2023), who in Iraq reported high *C. parvum* rates among cancer patients (90%) and hemodialysis ones (73.3%).

In the current study, the varied seasonal in human cryptosporidiosis infection rate increased in summer and autumn and fall or decreased in winter and spring. It was more common in the cases from rural areas than in urban ones and more in males than in females. Also, those used filtered water than those who used tap water very high infection rates. This agreed with Dyab *et al*. (2018) in Assuit Governorate, who noticed that seasonal variation in cryptosporidiosis infection rates that increased in summer (57.6%) and autumn (38.7%) and decreased in both winter (15.4%) and spring (31.2%), and that males showed high cryptosporidiosis (48.4%) than females (35.9%). However, it deviated from the present study in that urban regions had a higher infection rate (48.7%) than rural ones (26.1%). But, Farouk et *al*. (2021) in

Alexandria Governorate found that immunocompromised females showed more cryptosporidiosis than in males (60.9% vs. 51.9%), but without sexual differences among immunocompetent cases (32%).

In the present study, diarrhea showed high significant rate in immunocompromised patients than in immunocompetent persons, with higher parasites in diarrheal stool than in formed one. This agreed with Dong *et al*. (2020) in China, who reported that patients with gastrointestinal symptoms had higher *C. parvum* infection prevalence than asymptomatic people. Besides, Farouk *et al*. (2021) reported a substantial correlation between *C. parvum* and the diarrheal duration in immunocompromised patients. However, Shehata *et al*. (2019 in Alexandria didn't significant correlation between diarrhea and *C. parvum* infection hemodialysis patients as most of their immunocompromised patients suffered from HIV/AIDS.

In the present study, MZN stain for cryptosporidiosis showed that 21% of both groups were positive. This more or less agreed with Dyab *et al*. (2019), who found that by MZN 28% of 200 immunocompromised children were positive. But, the result disagreed with Farouk *et al*. (2021), who reported that by MZN-stained smears, 32% of immunocompetent cases and 56% of immunocompromised patients had cryptosporidiosis.

In the present study, as to microscopy diagnostic tests' sensitivity and specificity versus coproantigen ELISA, the mini-FLOTAC technique gave highest sensitivity (94%), followed by MZN staining after FECM with sensitivity (84%),but both iodine stained and direct smear examinations gave least sensitivity. The Mini-FLOTAC was superior in sensitivity than stained microscopic smears, and agreed with both Barda *et al.* (2013) in Italy and El-Nadi *et al.* (2019) in Upper Egypt. However, Chalmers *et al*. (2011) in the United Kingdom reported that MZN sensitivity was 75.4%, significantly lower than that for others as an immunochromatography lateral flow assay gave 84.9% (P = 0.0016), and specificities were 100% when the ICLF and EIA test algorithms included confirmation of positive reactions. Also, Tahvildar-Biderouni and Salehi (2014) in Iran reported a sensitivity of 94% and specificity of 100%. However, Gerace *et al*. (2019) in Italy found that with MZN stain several artifact structures stained as red spherical by microscope.

In the present study, coproantigen ELISA showed that the standardized immunoassay test is a straightforward, quick, dependable, and affordable modality that was more sensitive and specific. This agreed with both Massoud *et al.* (2008), & El-Settawy and Fathy (2012) in Egypt and Razakandrainibe *et al.* (2021) in France, who reported that sensitivity and specificity of coproantigen ELISA were 98.86% and 94.32%, respectively.

Conclusion

Cryptosporidium parvum is the commonest among immunocompromised patients and awareness about the mode of transmission and its diagnosis must be considered.

The mini-Flotac proved a valuable diagnostic test compared to coproantigen ELISA diagnosis. It has proved to be sensitive and specific, but time-consuming and needs an expertise than the coproantigen ELISA.

Water source filtration and treatment techniques should have more attention to cryptosporidiosis eradication.

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Authors' contribution: All the authors equally shared in field and theatrical work. They wrote the manuscript and approved its publication.

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Explanation of figures

Fig. 1: Oocysts 3-5µ rounded with red color on blue background in MZN stained stool (×100).
Fig. 2: Oocysts 3-5µ rounded with non-stained by Mini-flotac technique (×100).
Fig. 3: Copro-antigen ELISA oocysts yellow-colored wells and non-colored for negative ones.
Fig. 4: Comparison between immunocompromised and immunocompetent groups as to coproantigen ELISA