Original Article

Molecular and Microscopical Diagnosis of Cryptosporidial Infection among Immuno Compromised and Immuno Competent Patients

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Abstract

Objective: To study the prevalence of cryptosporidial infection among immuno compromised and immunocompetent patients by using microscopic and molecular examination. **Methods:** A hospital-based, cross-sectional study was conducted in the fevers hospital, Alexandria, Egypt. 300 individuals including 150 patients with immuno compromising conditions (90 with HIV/AIDS and 60 with renal failure and undergoing hemodialysis) and 150 immuno competent patients (meningitis, acute hepatitis, skin cellulitis and erysipelas) were enrolled in the present work. Stool samples were collected and subjected to modified Ziehl-Neelsen and nested PCR to detect cryptosporidiosis. EDTA blood samples of immuno compromised patients were collected for CD4+ T-cell counting. **Results:** *Cryptosporidium* infection rate among immuno competent patients was approximately half the rate detected among immuno compromised patients (32% *vs.* 56%) and the difference was statistically significant (P<0.001). Rural residence and illiteracy were found to be highly associated with *Cryptosporidium* spp. infection among both immuno-compromised and immuno-competent groups. 54% of infected patient with low CD4T cell count (<200) had moderate oocyst density and 21.7% of them had high cyst density while 50% of those with CD4 T cell count>200 showed low oocyst counts and only 5.3% of them presented with high oocysts density (P=0.001). Only 15 samples with high oocyst densities yielded amplicons. Compared to MZN, PCR showed a sensitivity of 11.4% and a specificity of 100%. **Conclusion:** DNA amplification may be inhibited due to the presence of substances as hemoglobin degradation products, bilirubin and bile acids in the feces leading to false-negative PCR results. Meanwhile, MZN staining smears showed enough accuracy for *Cryptosporidium* diagnosis.

Keywords: Cryptosporidial infection; Immuno compromised; Immuno competent patients; PCR; CD_4 T- Cells; Diarrhea

Introduction

Cryptosporidium species are opportunistic intracellular apicomplexan parasites that can cause self-limited diarrhea in healthy individuals, but life-threatening persistent diarrhea and other gastrointestinal disorders in infants, young children, and immuno compromised patients. [1-4] It is reported to be associated with patients suffering from AIDs or those under immunosuppressive drugs whereas it is responsible for significant morbidity and mortality in HIV/AIDS patients. [5] These species can have a zoonotic potential of transmission, where oocyst contamination of food and water from animal sources plays a key role in the transmission of the parasite. [6] Moreover, there is evidence of its transmission by inhalation, leading to respiratory cryptosporidiosis. [7] Meanwhile, fecal-oral route remains the main route of transmission of this parasite which is recognized as a highly infectious enteric pathogen. [8,9] Higher rates can be observed in developing countries (5% to 10%) as compared with developed countries (1% to 3%). [10]

Early detection of opportunistic intestinal parasites in immuno compromised patients has great validity to safeguard the patients against chronic morbidity, so they should be screened within reasonably safe intervals of time by examining stools samples using efficient diagnostic techniques. [7,11,12] Different methods can be used in the diagnosis of *Cryptosporidium* spp. including modified Ziehl-Neelsen Method (MZN), immunofluorescence staining and Polymerase Chain Reaction (PCR). MZN has different advantages such as low cost, screening a large number of samples, and permanent stain-making positive slides that can be used as a reference laboratory for confirmation. [13] Although,

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some limitations are reported including low sensitivity, low specificity and it requires experience in distinguishing *Cryptosporidium* oocysts from yeast. [14] On the other hand, PCR is more sensitive but time-consuming, expensive [13,15] and no large relative study has been performed to govern the ideal primers, PCR conditions, or stool extraction methods to use with clinical samples. [16]

In Egypt, previously published studies determined the prevalence of *Cryptosporidium* among different categories of immuno compromised patients to the genus level based on micro scopical examination of stained smears. [17-19] *Cryptosporidium* spp. was found to be the most predominant protozoan parasites among immuno compromised patients. For instance, rates of 60.2% (100/166) and 40% (60/150) were reported among immunosuppressed children from minia governorate and patients with chronic kidney disease undergoing hemodialysis in qena governorate, respectively. [18,19] Therefore, the present study aimed to detect *Cryptosporidium* oocysts based on based on using both microscopic and molecular techniques among and immuno competent patients in Alexandria, Egypt.

Materials and Methods

Study design, subject recruitment, and ethical considerations

A hospital-based, cross-sectional comparative study was conducted in the fevers Hospital, Alexandria, Egypt in the period from May 2019 to May 2021. The protocol of the study was reviewed and approved by the research ethics committee of the high institute of public health, Alexandria University. One-hundred and fifty immuno compromised patients (human immunodeficiency virus HIV/AIDS and RF patients undergoing hemodialysis) and 150 immuno competent patients (meningitis, acute hepatitis, skin cellulitis and erysipelas) were recruited after explaining to them the aim of the study and obtaining their written informed consent.

Data and sample collection

A structured questionnaire was used to collect data on demographic characteristics, clinical symptoms, and animal contact history. Double fresh stool samples were collected on 3 consequential days from each participant in a pre-labeled clean container, and a portion of the sample was stored in a sterile tube at -20℃ until the performance of DNA extraction. EDTA blood samples of immuno compromised patients with HIV/AIDS and RF were collected for CD4+ T-cell counting.

Sample analysis

Detection of *Cryptosporidium* oocysts: Stool samples were collected and transferred to be processed in the parasitology laboratory of the tropical health department in the high institute of public health, Alexandria University. These samples were concentrated using the formol-ethyl acetate sedimentation technique, and then two permanently stained smears were prepared from the sediment, fixed, and stained with the MZN technique according to standard procedures. [20,21] Stained smears were examined under the oil immersion lens of a light microscope, and the identification of acid-fast *Cryptosporidium* oocysts was done according to the diagnostic criteria and bench aids of the Center for Diseases Control and prevention (CDC). The intensity of infection was recorded by counting the number of oocysts per high power field (oocyst /HPF) in the positive cases (Mild infection:1-5 oocysts/ HPF, moderate infection: 6-10 oocysts/ HPF, and heavy infection ≥ 10 oocysts/ HPF). [22]

Counting CD4+T-cells: EDTA-blood samples were processed for CD4+T-cell counts within a maximum of two hours from the collection using the Attune™ NxT Flow Cytometer (Thermo Fisher Scientific, Waltham, MA, USA) in the hematology laboratory of the clinical pathology department at Alexandria University

Molecular detection and genotyping

DNA extraction: Total genomic DNA was extracted from stool samples, as about 200 mg of each pooled sample was subjected to spin column DNA extraction using i-genomic TM Stool DNA extraction mini kit (Cat. No 17451; iNtRON Biotechnology, Inc., Korea), according to the manufacturer's instructions. The eluted DNA was stored at -20℃ until the performance of PCR.

DNA amplification: Nested PCR was performed using a thermocycler (Beco, Germany) to amplify an 826-to-864 base pairs(bp) DNA fragment of the 18S small-subunit rRNA gene of *Cryptosporidium* species according to the protocol by Xiao et al. [23]

DNA detection and purification: DNA amplicons obtained by PCR were separated by electrophoresis in 1% (w/v) agarose gel stained with ethidium bromide that is added during the preparation of the gel, and bands were visualized using a UV transilluminator. DNA products were purified using gene JETTM PCR Purification Kit (Thermo ScientificTM K0701, USA), according to the manufacturer's instructions.

Statistical Analysis

Data were analyzed using IBM SPSS Statistics, version 20.0 (IBM Corp., Armonk, NY, USA). Categorical variables were compared using the chi-square test or Fisher's exact test, whichever is suitable. Differences and associations were considered statistically significant at P values <0.05.

Results

The present study included 150 patients with immunocompromising conditions (90 with HIV/AIDS and 60 with RF and undergoing hemodialysis) and 150 immunocompetent patients (meningitis, skin cellulitis and erysipelas, hepatitis). Males constituted 54% of immuno-compromised patients while both sexes were equally presented in the immuno-competent group. More than half (54%) of immuno-compromised patients and 70% of immune-competents were younger than 40 years. Urban residence was associated with immuno-compromising conditions as 96% of these patient were from urban areas versus 64% only of immune-competent individuals. The majority of immune-compromised cases were educated (74%) while 62% of immune-competent were illiterates, the difference was statistically significant. The higher percentages of both immune-compromised and immune-competent patients didn't work (58% and 62% respectively).

Only 38% of immune-compromised cases were suffering from diarrhea of whom 68.4% had prolonged diarrhea for one week and more. Also, 24% only of immune-competent patients were suffering from diarrhea but most of them (91.7%) confirmed that diarrhea duration didn't exceed one week. The differences were statistically significant. Out of the 150 immunocompromised patients 44% had CD4+ T-cell counts of <200/µl. HIV/AIDS was found mainly transmitted sexually (53.3%) followed by the medical and addiction ways (53.3%, 23.3% and 20% respectively) [**Table 1**].

Cryptosporidium oocysts were detected in 56% (84/150) of patients with immunocompromising conditions compared to 32% (48/150) of immune-competent group. A statistically significant differences have been found (P <0.001). Immuno-compromised females presented with slightly higher *Cryptosporidium* spp. infection rate than males (60.9% *vs.* 51.9%), while both sexes of immuno-competent group were equally infected (32% each). Almost equal rates of infection were observed for immuno-compromised patients younger than 40 ys. old and for older ones (55.6% and 56.5% respectively), while immune-competent patients older than 40 ys had a higher *Cryptosporidium* spp. rate than young individuals (40% *vs.* 28.6%). Rural residence and illiteracy were found to be highly associated with *Cryptosporidium* spp. infection among both immuno-compromised and immuno-competent groups (P<0.001). Non-working individuals in both groups were more infected with *Cryptosporidium* spp. (62.1% and 35.5% respectively) compared to those who had jobs in both groups (47.6% and 26.3% respectively), but without statistical significance. Diarrhea was associated with *Cryptosporidium* spp. infection among immuno-compromised cases as all diarrheic individuals were actually found infected. On the other hand, non- diarrheic immuno-competent patients suffered from higher rate of infection compared to diarrheic ones (36.8% *vs.* 16.7%, P=0.024). A significantly higher infection rate was observed among immuno-compromised patients that had CD4T cell counts <200 cells/ul when compared to those with higher cell count (69.7% *vs.* 45.2%, P<0.001) [**Table 2**].

Table 3 shows that 50% of the infected immunocompromised patients had moderate *Cryptosporidium* oocysts counts (5-9

Table 1: Distribution of characteristics among immune compromised and immune competent patients.

Variable	Immunocompromised patients (N=150)	Immunocompetent patients (N=150)	P-value
Gender	**n (%)**	**n (%)**	
Male	81 (54.0)	75 (50.0)	0.488
Female	69 (46.0)	75 (50.0)	
Age (years)			
<40	81 (54.0)	105 (70.0)	0.004*
≥ 40	69 (46.0)	45 (30.0)	
Residence			
Urban	144 (96.0)	96 (64.0)	<0.001*
Rural	6 (4.0)	54 (36.0)	
Education			
Illiterate	39 (26.0)	93 (62.0)	<0.001*
Educated	111 (74.0)	57 (38.0)	
Job			
Working	63 (42.0)	57 (38.0)	0.48
Not working	87 (58.0)	93 (62.0)	
Diarrhea			
Yes	57 (38.0)	36 (24.0)	0.009*
No	93 (62.0)	114 (76.0)	
Duration of diarrhea			
<one week	18/57 (31.6)	33/36 (91.7)	<0.001*
≥ one week	39/57 (68.4)	3/36 (8.3)	
Frequency of diarrhea			
<4 times/day	27/57 (47.4)	15/36 (41.7)	0.59
≥ 4times/day	30/57 (52.6)	21/36 (58.3)	
Level of CD4 count			
ε 200	84 (56.0)		
<200	66 (44.0)		
Cause of HIV transmission (N=90)			
Unknown	3 (3.3)		
Sexual	48 (53.3)		
Medical	21 (23.3)		
Addiction	18 (20.0)		

N: Number of individuals examined, NA: Not applicable, p: p value for Chi square test for comparing between the studied groups, *: Statistically significant at p ≤ 0.05

Table 2: Comparison of microscopy-based *Cryptosporidium* infection among immune compromised and immune competent patients.

Variable	*Cryptosporidium* spp						
	HIV/AIDS and RF patients			Healthy individuals			
	N	n (%)	P-value	N	n (%)	P-value	p_1
Overall	150	84 (56.0)		150	48 (32.0)	-	<0.001*
Gender							
Male	81	42 (51.9)	0.267	75	24 (32.0)	1	1
Female	69	42 (60.9)		75	24 (32.0)		
Age (years)							
<40	81	45(55.6)	0.905	105	30 (28.6)	0.169	0.319
≥ 40	69	39 (56.5)		45	18 (40.0)		
Residence							
Urban	144	78 (54.2)	Fe_p=0.035*	96	12 (12.5)	<0.001*	<0.001*
Rural	6	6 (100)		54	36 (66.7)		
Education							
Illiterate	39	33 (84.6)	<0.001*	93	39(41.9)	0.001*	<0.001*
Educated	111	51 (45.9)		57	9(15.8)		
Job							
Working	63	30 (47.6)	0.078	57	15(26.3)	0.243	0.603
Not working	87	54 (62.1)		93	33(35.5)		
Diarrhea							
Yes	57	57 (100)	<0.001*	36	6(16.7)	0.024*	<0.001*
No	93	27 (29)		114	42(36.8)		
Duration of diarrhea							
<one week	18	18 (100)	–	33	6(18.2)	Fe_p=1	0.002*
≥ one week	39	39 (100)		3	0(0.0)		
Frequency of diarrhea							
<4 times/day	27	27(100)	–	15	3(20.0)	Fe_p=0.677	Fe_p=1
≥ 4times/day	30	30(100)		21	3(14.3)		
Level of CD4 count							
≥ 00	84	38 (45.2)	<0.001*				
<200	66	46 (69.7)					

N: Number examined; n: Number of cases positive for Cryptosporidium oocysts; (-): not applicable; p: p value for Chi square test or Fisher Exact for comparing between absent and present infection in each group; p1: p value for Chi square test for comparing between the studied groups in presence of infection; *: Statistically significant at p ≤ 0.05

Table 3: Association between CD4+ T-cell counts and the density of *Cryptosporidium* oocysts among immunocompromised patients.

CD4+ T-cell count	N	Density of Cryptosporidium (oocysts/ HPF)				
		Total infected	Low <5	Moderate 5-9	High ≥ 10	P-value
		n	n (%*)	n (%*)	n (%*)	
≥ 200	84	38	19 (50.0)	17 (44.7)	2 (5.3)	
<200	66	46	11 (23.9)	25 (54.3)	10 (21.7)	0.001*
Total	150	84	30 (35.7)	42 (50.0)	12 (14.3)	

*: Calculated from total infected in each row; χ^2: Chi square test; p: p value for comparing between the studied categories; N: number examined. **: Statistically significant at p ≤ 0.05; n: number infected

oocysts/HPF) while 14.3% had high oocysts counts (>10 oocysts/HPF). Taking in consideration the CD4 T cell count, it was found that 54% of infected immuno-compromised patient with low CD4T cell count (<200) had moderate oocyst density, 21.7% of them had high cyst density while 23.9% had low density. On the other hand, 50% of the infected patients with CD4 T cell count >200 showed low oocyst counts while only 5.3% of them presented with high oocysts density. The differences were statistically significant.

Table 4 shows that about half of the diarrheic individuals had moderate oocyst count, while 23.8% of them had either low or high counts. This was evident for diarrheic HIV patients as 52.9% had counts of 5-9 oocysts/HPF, 23.5% of them had low count and the remaining 23.5% had high count. Considering diarrheic RF patients had all a moderate oocyst count. On the other hand, half the immuno-competent patients presented either by low density or high density oocysts. The differences were statistically significant.

MZN staining technique identified 132 positive samples out of the 300 samples examined (44%), only 15 samples of them had high oocyst densities and yielded amplicons that were detected as positive by PCR (5%), the difference was statistically significant. As regards HIV patients, 73.3% of them were positive by MZN technique while PCR identified 13.3% only positive cases among them. All infected RF (30%) patients were identified by MZN technique only. The percentages of positive samples of immuno-competent patient were 32% and

Table 4: Association between the density of *Cryptosporidium* oocysts and diarrhea among immunocompromised and immunocompetent patients.

Intensity of infection	Total diarrheic n (%)	Diarrhea			MCP-value
		HIV	RF	Immunocompetant	
		n (%)$	n (%)$	n (%) $	
<5	15 (23.8)	12 (23.5)	0 (0.0)	3 (50)	0.006*
5–9	33 (52.4)	27 (52.9)	6 (100)	0 (0.0)	
≥ 10	15 (23.8)	12 (23.5)	0 (0.0)	3 (50)	
Total	63	51	6	6	

$: Calculated from the total of each column; MC: Monte C; p: p value for comparing between the studied groups; *: Statistically significant at p ≤ 0.05

Table 5: Detection of *Cryptosporidium* by PCR versus MZN among immunocompromised and immunocompetent patients.

Type of patient	Total examined	PCR N (%)	MZN N (%)	P-value
HIV/AIDS	90	12 (13.3)	66 (73.3)	
RF	60	0 (0.0)	18 (30.0)	<0.001*
Immunocompetent	150	3 (2.0)	48 (32.0)	
Total sample	300	15 (5.0)	132 (44.0)	
Sensitivity$		11.40%		
Specificity$		100%		
PPV$		100%		
NPV$		59%		

χ2: Chi square test; MC: Monte C; p: p value for comparing between the studied groups; *: Statistically significant at p ≤ 0.05; $: MZN used as a gold standard test

2% for MZN and PCR respectively. So compared to MZN, PCR revealed a sensitivity of 11.4% and 100% specificity [**Table 5**].

Discussion

Since the first report on the association of *Cryptosporidium* species with overwhelming watery diarrhea in a patient immunosuppressed with drugs in the mid-1970s, [24] they became one of the major diarrhea-causing pathogens among immunocompromised patients, infants, and young children. Apart from the detection of *Cryptosporidium* oocysts using staining and microscopy, understanding the role of zoonotic transmission of infection in different geographic areas requires the use of molecular tools. [25] Up to the best of our knowledge, this is the first molecular detection of *Cryptosporidium* among patients with HIV/AIDA and RF in Alexandria, Egypt.

Microscopy of MZN-stained smears revealed that more than half of patients with immunocompromising conditions 56% (73.3% of HIV and 30% of RF patients) and about a third of immunocompetent individuals 32% were infected with *Cryptosporidium* spp. A study carried out in Thailand revealed that HIV patients harbored a high rate of cryptosporidiosis (67.1%). [16] A rate of 40.0% among chronic kidney disease patients undergoing hemodialysis and 6.0% among healthy controls were recently reported from Qena. [17] Lower rates of *Cryptosporidium* spp. were detected among HIV patients in Iran and India (1.5% and 2% respectively). [26,27] Also, in Uganda and Zambia, it was reported that the overall prevalence was 2.17% among Anti-Retroviral Therapy (ART) HIV/AIDS patients and 9.5% among adult HIV individuals in contact with livestock. [28,29] Meta-analysis of *Cryptosporidium* infection among HIV individuals identified 270 studies with 89,724 patients from 59 countries examined revealed that the overall prevalence was 8.69%. [5]

The present study detected equal *Cryptosporidium* infection rates among the immunocompetent males and females (32%), while immunocompromised females showed a slightly non-significant higher rate than males (60.9% *vs.* 51.9%). In contrast, Bednarska et al. [30] revealed a statistically significant difference (P=0.015) in the rate of infection with *Cryptosporidium*, giardia, and cyclospora between the male and female immunodeficiency patients (5% and 0.6% respectively).

The significant association of *Cryptosporidium* species with diarrhea and its duration among patients with immunocompromising conditions in the present study is in agree with that reported by Bednarska et al. [30] who demonstrated that the infection with *Cryptosporidium* spp. was significantly associated with diarrhea especially among heavily immunodeficient patients (p=0.002). In contrast, Shehata et al. [31] didn't find any significant association between infection with *Cryptosporidium* species and diarrhea among hemodialysis patients from Alexandria. However, this difference could be attributed to the fact that many of the immunocompromised patients in the present were HIV/AIDS patients. The role of the density of oocysts in the presentation of diarrhea could not be ruled out, as higher oocyst intensities were significantly associated with diarrhea in the present study (76.4% of diarrheic patients had an oocysts count >5 oocysts/HPF). Moreover, the majority of infected immunocompromised patients with CD4+ T-cell counts <200 cells/µl showed higher oocysts densities (>5). In agreement with the finding of the present study, the results done in upper Egypt that revealed a correlation between infection with *Cryptosporidium* spp. and diarrhea among

chronic renal failure patients undergoing hemodialysis. [17] In this context, several African studies link persistent diarrhea to cryptosporidiosis among children suffering from HIV/AIDS infection. [32]

The actual study reported a strong association (P<0.001) between infection with *Cryptosporidium* spp. and the reduction in the CD4+ T-cell counts (<200 cells/µl) among patients with immunocompromising conditions, and this was consistent with Ghafari and his colleagues (P=0.000) among HIV/AIDS infected patients in Iran and among hemodialysis patients in Alexandria. [31,32] Moreover, it was in agreement with results reported among hemodialysis patients from Saudi Arabia and HIV-infected patients in Ethiopia and Cameroon. [33-35]

In present study, a high rate of *Cryptosporidium* spp. was detected by MZN as compared to molecular technique (PCR) 44% (132/300) and 5% (15/300) respectively, the difference was statistically significantly. PCR showed a sensitivity of 11.4% and 100% specificity compared to MZN. In contrast to our results, several studies reported molecular techniques to be more sensitive and specific than traditional detection techniques. Aghamolaie detected cryptosporidiosis among diarrheic children using MZN microscopic method, DFA assay, and PCR analysis, positive rates of 1.2%, 1.1% and 1.27% were recorded by the three techniques respectively. Nested-PCR assay showed higher sensitivity and specificity (100% each) as compared to MZN (94% and 100%) and DFA (87.5% and 100%). [36] In the southwest of Iran, 2018, Ghafari et al. [32] detected *Cryptosporidium* infection rates of 7.2% and 10.8% among HIV/AIDS patients using MZN and nested-PCR-RFLP technique respectively. Zhao et al. [37] showed high sensitivity of PCR in identifying *Cryptosporidium* species, Uppal et al. [38] reported also that PCR was more sensitive than MZN and ELISA technique but required a more hands-on time per sample and was more expensive than microscopy. On the other hand, some researchers tried to explain the false negative results detected by molecular techniques and hence their lower sensitivity. Lantz et al. [39] described that the presence of hemoglobin degradation products, bilirubin, and bile acids in the feces may inhibit DNA amplification and could lead to false-negative PCR results. In addition, the inefficient methods for isolating organisms when present in small number in samples and resistance of the organism to disruption and lysis could be also the causes of lowering the sensitivity. [40] In 2018, Hedman et al. [41] revealed that the exact mechanisms of DNA polymerase inhibitors have not been explained. Additionally, for many molecules, the mechanism of inhibition has not been elucidated, *i.e.* whether they affect the ion content, nucleic acids, or DNA polymerase activity.

Conclusion

Our findings confirm the high susceptibility of patients with immunocompromising conditions to infection with different *Cryptosporidium* spp. compared to immunocompetent patients. Significant association of *Cryptosporidium* spp. with rural residence, illiteracy, diarrhea and CD4+ T-cell counts <200 cells/µl was proofed. Some substances such as hemoglobin degradation products, bilirubin, and bile acids in the feces might inhibit DNA amplification leading to false-negative PCR results. Regarding the possibility of low PCR sensitivity, its costs and its unavailability in many laboratories and hospitals and as MZN staining has enough accuracy, MZN remains the gold standard technique for *Cryptosporidium* diagnosis.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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