Original Article

Cryptosporidiosis in patients with diarrhea and chronic liver diseases

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Abstract

Introduction: The aim of this study was to evaluate the epidemiology and clinical significance of *Cryptosporidium* in patients with diarrhea and chronic liver diseases.

Methodology: The study included 150 patients with chronic liver diseases and diarrhea, and 50 subjects with diarrhea as a control group. Stool samples were screened for the presence of *Cryptosporidium* by microscopic examination after modified Ziehl-Neelsen staining and detection of *Cryptosporidium* coproantigen by enzyme-linked immunosorbent assay (ELISA).

Results: The prevalence of *Cryptosporidium* infection in patients with chronic liver diseases was 30% (45/150) versus 14% (7/50) in controls. *Cryptosporidium* infection increased with the progression of chronic liver diseases from Child-Pugh class A to Child-Pugh class C ($p< 0.001$) and from model for end-stage liver disease (MELD) score ≤ 9 to MELD score > 9 ($p< 0.031$). Nine patients in Child-Pugh class C with diarrhea associated with *Cryptosporidium* infection developed hepatic encephalopathy, and only diarrhea was identified as a precipitating factor for hepatic encephalopathy.

Conclusions: *Cryptosporidium* is one of the important causes of diarrhea in patients with chronic liver diseases. The infection significantly increased with the progression of chronic liver diseases. In patients with advanced chronic liver diseases, *Cryptosporidium* infection may be a precipitating factor of hepatic encephalopathy.

Key words: *Cryptosporidium*; liver diseases; diarrhea, hepatic encephalopathy.

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Introduction

The intestinal coccidian parasite *Cryptosporidium* has emerged as a significant human pathogen worldwide [1]. Globally, *Cryptosporidium* infection continues to be a significant health problem recognized to be an important cause of diarrhea in both immunocompromised and immunocompetent people [2,3]. It is an obligate intracellular extracytoplasmic protozoan parasite that is a major cause of diarrheal illness worldwide [4]. In developing countries, *Cryptosporidium* infection occurs mostly in children younger than five years of age and in AIDS patients [5].

C. hominis and *C. parvum* are responsible for the majority of human infections. *C. hominis* is found almost exclusively in humans, whereas *C. parvum* is found also in domestic livestock and wild animals. *C. parvum*, the most common cause of cryptosporidiosisin humans, can be divided into two major genotypes. These two genotypes, human and bovine, are also referred to as genotype 1 and genotype 2, respectively [6,7]. Although *Cryptosporidium* organisms are known to be zoonotic pathogens, human-to-human transmission seems to be the most common way of transmission, through the fecal-oral route, by the ingestion of *Cryptosporidium* oocysts through the consumption of food, drink, or recreational water contaminated with fecal matter [8,9].

Cell-mediated immunity appears to be the major component of the immune response to *Cryptosporidium* infection [10]. Patients with chronic liver failure, who have an impaired immune status, are often highly susceptible to infection [11]. Laboratory diagnosis of cryptosporidiosis is usually achieved by microscopic detection of *Cryptosporidium* oocysts in

stool specimens and staining techniques including acid-fast stains and immunofluorescence [12]. Little is known about the *Cryptosporidium* infection in chronic liver diseases and hepatocellular carcinoma, so this study aimed to elucidate the prevalence and clinical significance of *Cryptosporidium* infection in patients with diarrhea and chronic liver diseases.

Methodology

Patients

This study was carried out on 150 patients (71 males and 79 females) with diarrhea and chronic liver disease between 3 September 2012 and 12 October 2013. Fifty subjects (32 males and 18 females) with diarrhea, free of any known liver diseases, matched for ethnicity, sex, and age, were recruited as a control group. The diagnosis of hepatitis C virus (HCV) was made by positive anti-HCV and reverse transcription polymerase chain reaction (PCR-RT), and the diagnosis of hepatitis B virus (HBV) was made by positive hepatitis B surface antigen (HBsAg) and PCR. Liver cirrhosis was diagnosed by history, clinical examination, abnormal liver function tests, abdominal ultrasound, and liver biopsy. Ascites was diagnosed by clinical examination, abdominal ultrasound, and abdominal computerized tomography (CT). Hepatocellular carcinoma (HCC) was diagnosed by abdominal ultrasound, triphasic CT, and elevated serum level of alpha fetoprotein.

All known causes of liver diseases were excluded based on analytical, clinical, and epidemiological data; control subjects were negative for both serum anti-HCV and HBsAg. Autoimmunity, metabolic, genetic disorders, nonalcoholic steatohepatitis (NASH), alcohol intake, clinical or biochemical history of acute hepatitis, and drug toxicity were excluded, and all cases were negative for anti-HIV antibodies. In brief, participants were recruited from three outpatient clinics and two centers in Mansoura University Hospital (Tropical Medicine Department, Clinical oncology and Nuclear medicine Department, and Internal Medicine Department). The study conformed to the medical ethics of the 1975 Declaration of Helsinki, and all participants provided written informed consent. Diarrhea was defined as three or more unformed stools in a 24-hour period. All hepatic patients using lactulose were excluded, to avoid the laxative effect. Also, all patients with immunosuppression (*e.g.*, diabetes mellitus and HIV infection) were excluded.

Initially, the study populations were classified into four groups. Group A comprised 50 patients with liver cirrhosis without ascites, 46 patients with HCV-related infections, and 4 patients HBV-related infections. Group B comprised 50 patients with liver cirrhosis and ascites, 48 patients with HCV-related infections, and 2 patients with HBV-related infections. Group C comprised 50 patients with hepatocellular carcinoma (HCC), 44 patients with HCV-related infections, 2 patients with HBV-related infections, and 4 patients with combined HCV and HBV infections. None of the patients with HCC were receiving treatment (*e.g.*, sorafenib and/or transarterial chemoembolization [TACE]). The diagnosis of HCC was made according to the Barcelona-2000 conference on the clinical management of hepatocellular carcinoma [13]. Group D comprised 50 individuals free of any liver diseases and complaining of diarrhea with matching age and sex as a control group.

All patients and controls included in the study were subjected to thorough clinical history, with the following points noted: age and sex, loss of weight, jaundice, vomiting, diarrhea and its characteristics, abdominal pain, right hypochondrial pain, dyspnea, cough and expectoration, discoloration of urine, history of drug intake of corticosteroids and cytotoxic drugs, and symptoms related to other body system affections. Full family and medical history of previous episodes of diarrhea, potential exposure to food poisoning, andgeneral and abdominal examination included imaging using an ultrasound scan and triphasic CT were done.

Assessment of the severity of the liver diseases

Since patients with HCC can present with or without ascites, showing an overlap with groups A and B, the patients were further classified according to the modified Child-Pugh and model for end-stage liver disease (MELD) score to access the correlation between *Cryptosporidium* infection and the severity of liver diseases.The Child-Pugh score consists of five clinical features and is used to assess the prognosis of chronic liver diseases based on the degree of ascites, the plasma concentrations of bilirubin and albumin, the prothrombin time, and the degree of encephalopathy. The Child-Pugh score corresponds to the total points for each item. According to the sum of these points, patients can be categorized into Child-Pugh grades. A total score of 5 to 6 is considered grade A (well-compensated disease); 7 to 9 is grade B (significant functional compromise); and 10 to 15 is grade C (decompensated disease) [14]. The MELD score was calculated using the original formula without including the cause of liver disease [15]:

MELD score = 0.957 × $\log_e$ (creatinine mg/dL) + 0.378 × $\log_e$ (bilirubin mg/dL) + 1.120 × $\log_e$ (international normalized ratio [INR]) + 6.43. The MELD score calculator from the UNOS website (www.unos.org) was used.

Laboratory investigations and serological tests

Fasting (12 hours) serum and plasma samples were obtained to test serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), α-fetoprotein (Liaison AFP kit, DiaSorin, Vercelli, Italy). HBV, HCV, and HIV were screened using an enzyme-linked immunosorbent assay (ELISA) technique (INNOTEST HCV Ab IV; Fujirebio Europe N.V, Ghent, Belgium) 100% sensitivity and 100% specificity, respectively (National Serologic Reference Laboratory, Australia; http://www.nrl.gov.au). The laboratory investigationswere done to exclude other diseases such as diabetes mellitus and other malignant diseases such as leukemia by using standard clinical chemistry analyzers. Stools were examined by direct smear for parasitic diseases.

Collection of fecal samples

Three stool samples from each patient in the study were collected to increase sensitivity. Each individual in the study was supplied with two containers, one for direct examination by concentration method [16] and for staining with modified Ziehl-Neelsen stain [17]. The second container was preserved for detection of *Cryptosporidium* coproantigen by ELISA. Fecal samples were collected in dry, clean, labeled plastic containers with tight-fitting covers containing no preservatives; stool contamination was avoided. Macroscopic examination was first conducted focusing on several aspects: consistency, presence of blood and mucus, and macroscopic parasitic elements. Microscopic examination was done using a direct wet smear technique to test for the presence of oocysts of parasitic protozoa [18].

The *Cryptosporidium* antigen was detected in the stool by ELISA. The method was performed according to the instructions supplied with the commercial ELISA kit (Ridascreen Cryptosporidium, C1201, R-Biopharm AG, Germany).

Molecular diagnosis of the species

DNA extraction was performed using the QIAamp DNAstool kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Species identification was carried out bynested amplification of the *Cryptosporidium* oocyst wallprotein (COWP) gene followed by RsaI restriction enzymedigestion [19]. Thedigestion pattern of the amplicon fragment (543–554 bp) for *Cryptosporidiumparvum* is 34, 106 and 413 bp fragments,and for *Cryptosporidiumhominis* is 34, 106, 130 and 284 bp fragments.

Statistical analysis

A computer program, SPSS for Windows version 11.5.0, was used for data analysis. The descriptive data were given in mean (± SD). The Chi-square test, Student's t-test, and Fischer's bicaudal exact test were used for the analytical assessment. The differences were considered to be statistically significant when the p value obtained was less than 0.05.

Results

Clinical and epidemiological characteristics of Cryptosporidium infection in study populations

There was no statistically significant difference between both groups in terms of age and sex.Statistical analysis of the epidemiological data revealed that tap water was the only source of drinking water in the chronic liver diseases and control groups, with no significant difference. Also, no significant differences were found between both groups in terms of direct contact with pets ($p = 0.72$) and direct contact with sheep/cows ($p = 0.73$). Vomiting was more significant in the chronic liver diseases group than in the control group ($p < 0.05$), while there was no difference in terms of fever ($p = 0.48$). The number of bowel movements per day varied from three to ten. The duration of diarrhea ranged from three to six days, and all patients with *Cryptosporidium* infection showed no intractable diarrhea in both groups (Table 1). Nine patients with diarrhea associated with *Cryptosporidium* infection developed hepatic encephalopathy; all were class CChild-Pugh score. Four patients with liver cirrhosis with ascites developed hepatic encephalopathy (grade 1-2), five patients with HCC developed hepatic encephalopathy (grade 1-2), while no patients with liver cirrhosis without ascites developed hepatic encephalopathy. Only diarrhea was identified as a risk factor for the development of hepatic encephalopathy in patients after the exclusion of other precipitating factors (*e.g.*, gastrointestinal bleed, hyponatremia, and hypokalemia). Nitazoxanide is the only broad-spectrum antiparasitic drug that has been approved in the United States for treatment of cryptosporidiosis [20].

Table 1. Clinical and epidemiologic characteristics of *Cryptosporidium* infection in patients with chronic liver diseases and controls

	Chronic liver diseases (n = 150)	Controls (n = 50)	P value
	No. (%)	No. (%)	
Characteristics			
Age (years)(mean ± SD)	56.5 ± 7.4	56.5 ± 5.7	0.49
Sex (male : female)	71 : 79	32 : 18	0.50
Clinical features			
Associated fever	19 (12.6%)	5 (10%)	0.48
Associated vomiting	16 (10.56%)	3 (6.0%)	< 0.05
Water source (tap water)	150 (100%)	50 (100%)	NS
Direct contact with pets	54 (36%)	16 (32%)	0.72
Direct contact with sheep/cows	68 (45%)	15 (36%)	0.73

Table 2. Cryptosporidium infection in patient groups and controls

	Positive cases		
Groups	**No.**	**%**	**P value**
A: Liver cirrhosis without ascites (n = 50)	11	22	A vs B = 0.19 A vs C = 0.36
B: Liver cirrhosis with ascites(n = 50)	18	36	A vs E = 0.43
C: Hepatocellular carcinoma (n = 50)	16	32	B vs C = 0.8
D: Total chronic liver diseases (n = 150)	45	30	B vs E = 0.02 C vs E = 0.06
E: Controls (n = 50)	7	14	D vs E = 0.04

Table 3. *Cryptosporidium* infection

	Number of +ve cases	% of +ve cases	P value
Child-Pugh class			
A (n = 29)	4	10	
B (n = 38)	6	15.8	< 0.001
C (n = 72)	35	48.6	
MELD Score			
MELD ≤ 9 (n = 72)	16	22.22	0.031
MELD > 9 (n = 78)	30	38.5	

Table 4. Comparison between results of examination of acid-fast stained smears and antigen detection of *Cryptosporidium*

	AFS detection in 200 subjects		Total	Ag detection in 200 subjects		Total
	–ve	+ve		–ve	+ve	
Number	148	52	200	147	53	200
%	74%	26%	100%	73.5%	26.5%	100%
AFS –ve				147	1	
AFS +ve				0	52	

AFS: acid-fast stained; Ag: antigen

Table 5. Biochemical characteristics in the study populations

	Positive cases of *Cryptosporidium*	Negative cases of *Cryptosporidium*	P value
ALT (iu/L)	41 ± 37.5	49 ± 53.4	0.16
AST (iu/L)	38 ± 26.98	51 ± 22.96	0.09
Albumin (g/dL)	3.1 ± 0.5	3.3 ± 0.5	< 0.05
Bilirubin (mg/dL)	1.90 ± 0.97	1.30 ± 0.92	< 0.001
AFP (ng/mL)	8.64 ± 3.18	4.56 ± .81	0.001
INR	1.7 ± 0.37	1.4 ± 0.33	< 0.001
Creatinine (mg/dL)	1.4 ± 0.5	0.9 ± 0.9	< 0.05

ALT: alanine aminotransferase; AST: aspartate aminotransferase; AFP: alpha-fetoprotein

In this study, in addition to fluid and electrolyte replacement, nitazoxanide 500 mg twice daily for three days was used for treatment of all positive cases of cryptosporidiosis. All patients recovered from hepatic encephalopathy.In this study, other parasitic causes of diarrhea in patients with chronic liver diseases were found, such as *Entamoeba histolytica* in 3.9% (6/150), *Giardia lamblia* in 5.3% (8/150), *Strongyloides stercoralis* in 1.33% (2/150), and mixed infection by *Cryptosporidium* and *Giardia lamblia* in 6.6% (10/150). In the control group, *Entamoeba histolytica* was found in 6% (3/50), *Giardia lamblia* in 4% (2/50), *Strongyloides stercoralis* in 2% (1/50), and mixed infection by *Cryptosporidium* and *Giardia lamblia* in 6% (3/50). However, all parasitic causes of diarrhea other than *Cryptosporidium* were excluded, as the focus was on only *Cryptosporidium* in this study.

Cryptosporidium *infection in patients groups and controls*

The prevalence of *Cryptosporidium* infection in patients with liver cirrhosis without ascites was 22% (11/50), in patients with liver cirrhosis with ascites 36% (18/50), while the prevalence in patients with hepatocellular carcinoma was 32% (16/50) and in controls with diarrhea was 14% (7/50). The prevalence of *Cryptosporidium* infection was significant in liver cirrhosis with ascites versus the control group ($p = 0.02$). The results showed that the prevalence of positive *Cryptosporidium* infection in patients with chronic liver diseases was significantly higher than that in controls ($p = 0.04$) (Table 2). The prevalence of *Cryptosporidium* infection by species was 65.0% for *C. parvum* and 35% for *C. hominis*, including five mixed infections.

Cryptosporidium *infection according to the severity of the liver diseases*

When the patients with chronic liver diseases were classified based on their Child-Pugh score, the prevalence of *Cryptosporidium* infection increased progression of chronic liver diseases as demonstrated by a significant increase of the rate of infection from Child-Pugh class A to Child-Pugh class C ($p < 0.001$) (Table 3A) and according to MELD score, from a MELD score ≤ 9 to a MELD score > 9 ($p < 0.031$) (Table 3B).

Comparison between the results of examination of acid-fast stained smears and antigen detection for Cryptosporidium

Using *Cryptosporidium* coproantigen detection, *Cryptosporidium* was detected in 53 (26.5%) of the 200 samples included in this study. Using an acid-fast stain, *Cryptosporidium* was detected in 52 (26%) of the 200 samples included in this study. Comparison between the results of antigen detection and examination of acid-fast stained smears for *Cryptosporidium* revealed that one sample was positive by antigen detection and negative by microscopic examination of acid-fast smears (Table 4).

Biochemical characteristics of the study populations

No significant differences were found in serum liver enzymes between patients with and without *Cryptosporidium* infection. Patients with *Cryptosporidium* infection showed a significant decrease in serum albumin and a significant increase in serum bilirubin, INR, creatinine, and alpha-fetoprotein versus patients without *Cryptosporidium* ($p < 0.05$, $p < 0.001$, $p < 0.05$, and $p < 0.001$, respectively) (Table 5).

Discussion

Infection by *Cryptosporidium* has been reported across the globe and identified in patients from 3 days to 95 years of age [21]. Shrestha *et al*. (1993) found that oocysts of *Cryptosporidium* were detected in the stools of 20% of patients with liver disease [22]. In Egypt, *Cryptosporidium parvum* prevalence was found to range from 0%–47%. The risk factors for this protozoa, including population, ecology, and environmental findings, suggest that zoonotic transmission and water transmission modalities are the principal routes of infection [23]. Recently Badawy *et al*. (2012) reported a 31.1% prevalence of *Cryptosporidiumparvum* among Egyptian military recruits [24]. Previous studies demonstrated that severe liver injury and liver failure are closely associated with reduced cellular immunity [25]. Reduced cellular immune function could contribute to an increased susceptibility to infection in subjects with chronic liver diseases, including infection with *Cryptosporidium*. In this study, we found that 32% of patients with hepatocellular carcinoma and diarrhea harbored *Cryptosporidium*, compared to 22% in patients with liver cirrhosis without ascites and 36% in patients with liver cirrhosis with ascites.

In AIDS patients, *Cryptosporidium* infection can promote disease progression and worsen the prognosis of the disease [26]. Unlike the negative effect *Cryptosporidium* infection has on AIDS patients, our results demonstrated that *Cryptosporidium* infection does not appear to be problematic in patients with chronic liver diseases, as five patients (10%) with HCC and four patients (8%) with liver cirrhosis with ascites developed hepatic encephalopathy (grade 1-2), and all patients recovered from hepatic encephalopathy. This result in agreement with that of Hunter and Nichols (2002), who found that in cancer patients (other than those with hematological malignancies), cryptosporidiosis did not appear to be as problematic as it was in hematological malignancies [27]. In this study, *Cryptosporidium* infection increased with progression of chronic liver diseases from Child-Pugh class A to Child-Pugh class C. This result may be explained by the impaired cellular immune response in patients with chronic liver disease [25]. Several studies investigated the epidemiology and prognosis of *Cryptosporidium* infection in patients with malignant disease. Tanyuksel *et al*. (1995) reported that *Cryptosporidium* oocysts were detected in 18 (17.0%) of 106 patients with diarrhea and various cancers [28]. On the other hand, a low prevalence of *Cryptosporidium* was found in an Indian study that involved in 560 patients with cancer and diarrhea; oocysts were found in only seven patients (1.3%). Of these seven patients, five had hematological cancers [29]. In our study, a high prevalence of *Cryptosporidium* oocysts was found in our malignant patients with hepatocellular carcinoma (32%). The high prevalence found in our study compared to the prevalence found in other studies could be explained by differences in subject selection, as we selected only malignant patients with hepatocellular carcinoma rather than those with hematological malignancy. In addition, the high prevalence of *Cryptosporidium* infection in our patients suggests that these protozoaare widespread in our environment. This high prevalence of infections with these opportunistic parasites among patients with cancer may result from the patients' reduced immunity [30].

C. hominis and *C. parvum* are the most common species in humans [31]. Our study showed a predominance *C. parvum* over *C. hominis*. This result was in agreement with the results of another study in Egypt, where the predominant species were *C. parvum* over *C. hominis* [32], but contradictory to the results of another study in Egypt that showed an elevated prevalence of *C. hominis* over *C. parvum* [33]. Regarding the method of detection of *Cryptosporidium* infection, in the present work, both Ziehl-Neelsen stain and coproantigen detection methods were used. The number of positive cases was 53 (26.5%) by the antigen detection test and 52 (26%) by the Ziehl-Neelsen stain method. In accordance with these results, Bialek *et al*. (2002) found that the sensitivity of antigen enzyme immunoassay (EIA) and direct fluorescent antibody (DFA) were similar (94% and 95%, respectively) [34].

Conclusions

Cryptosporidium is an important cause of diarrhea in patients with chronic liver diseases. The infection significantly increases with progression of chronic liver diseases. In patients with advanced chronic liver diseases, *Cryptosporidium* infection may be a precipitating factor for hepatic encephalopathy. The clinical significance of this parasitic infection in patients with diarrhea and chronic liver diseases requires further study.

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