Medical Parasitology

Genotypic Prevalence of *Cryptosporidium* in Egyptian Patients with Liver Cirrhosis

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ABSTRACT

Background: Liver cirrhotic patients usually suffer from compromised immunity which makes them more susceptible to pathogens including *Cryptosporidium.*

Objective: To detect the genotypic prevalence of *Cryptosporidium* species among cirrhotic liver Egyptians. Also, to explore the association between the parasite and grades of liver cirrhosis and related patient's data.

Material and Methods: From September 2015 to January 2017, a cross-sectional study was carried out on a study group of 60 cirrhotic patients (HCV and HBV positive) attending the outpatient clinics of Hepatology, Gastroenterology, and Infectious diseases Department, Al-Azhar University Hospitals, Cairo, as well as a control matched group of 60 subjects. Collected stool samples were examined coproscopically and using the copro-nPCR assay.

Results: Only *Cryptosporidium parvum* was molecularly detected in 3.3% of liver cirrhotic patients among patients with HCV, suffering from hepatocellular carcinoma (HCC) and grade C liver cirrhosis. None of them were microscopically detected. The mean age of liver cirrhotic patients and controls was 46 years. None of the studied demographic, environmental or clinical data showed statistically significant association with molecular detection of *Cryptosporidium* (P-value >0.05). *Cryptosporidium* was not detected in any of the control groups.

Conclusion: There is a low prevalence of *Cryptosporidium* parvum among patients with progressive liver cirrhosis (suffering from HCC and with grade C cirrhosis). Copro-nPCR is more sensitive than coproscopy for the detection of *Cryptosporidium* infection. Our findings indicate the need for molecular searching of *Cryptosporidium* in this population to avoid delay in its final diagnosis.

Keywords: *Cryptosporidium;* Liver cirrhosis; Copro-nPCR; Prevalence.

INTRODUCTION

Cryptosporidium is a protozoan parasite that infects the epithelial cells of the small intestine causing diarrheal illness in humans. It has a worldwide distribution and is considered an emerging zoonotic parasite with reservoirs in cattle, domestic animals and in fecally contaminated environments.[1]

Transmission via the fecal-oral route is due to the ingestion of *Cryptosporidium* oocysts through the consumption of contaminated food or water and through direct person to person or animal-to-person contact. These small-sized oocysts (4-6 μm) are highly resistant to common household disinfectants.[2,3]
Diarrhea caused by *Cryptosporidium* is usually mild and self-limited in patients with good immunity.

However, it can become severe, long-standing, and life-threatening diarrhea in immunocompromised patients.[4]

Previous studies demonstrated that severe liver injury and liver failure are closely associated with reduced cellular immunity.[5] In one study 32% of patients with hepatocellular carcinoma and diarrhea harbored cryptosporidial infection, compared to 22% among patients who had liver cirrhosis without ascites and 36% in patients who had liver cirrhosis with ascites.[6] The diarrhea is associated with multiple infectious agents; thus, *Cryptosporidium* diagnosis should rely on laboratory investigations. Despite the major advances in diagnostic techniques, routine cryptosporidial infection diagnosis is still lacking in most clinical laboratories.[7] Based on the characteristic morphology of the oocysts, few microscopic techniques are commonly used for *Cryptosporidium* detection in feces. However, a complex clinical specimen like feces makes identification a challenging task. It is also time-consuming, tedious and requires great experience.[8]

Neither microscopy nor coproantigen assays can achieve that purpose. Therefore, many PCR-based assays were developed to detect and characterize *Cryptosporidium* DNA in feces.[9]

The use of molecular epidemiologic tools has provided new insights into the diversity of *Cryptosporidium* infection among humans and animals.[10] So far, 26 *Cryptosporidium spp* have been described.[11] Most human cases are caused by *C.hominis* and *C. parvum*.[10] Several other species are seen in humans at a lower frequency, including *C. meleagridis*, *C.felis*, *C. canis*, *C. ubiquitum*, and *C. cuniculus*.[10] Another new species, *C. viatorum*, has been described in 10 travelers returning to Great Britain from the Indian subcontinent at 2010.[12]

This study was done for detection of the molecular prevalence and genotyping of *Cryptosporidium spp.* in fecal samples of Egyptian patients suffering from liver cirrhosis. Exploration of the possibility of an association between *Cryptosporidium spp* and grades of liver cirrhosis and assessment of their demographic, environmental and clinical data for risk of infection was done.

MATERIAL AND METHODS

Study population

This cross-sectional study was carried out on a study group of 60 patients (33 males and 27 females) with liver cirrhosis (HCV and HBV positive), and a control group of 60 subjects (32 males and 28 females) free of any known liver diseases as a control group, matched for ethnicity, sex, and age.

Stool samples were collected from September 2015 to January 2017.

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 patient history, clinical examination, abnormal liver function tests, and abdominal ultrasound. The patients were further classified according to the modified Child-Pugh score, which consists of five clinical features, and was used to assess the prognosis of liver cirrhosis 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 three 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).

Exclusion Criteria: All known causes of liver diseases rather than (HCV and HBV infection) as well as patients suffering from immunosuppressive diseases (*e.g.*, diabetes mellitus and HIV infection) or with comorbidity were excluded based on analytical, clinical, and epidemiological data.

Participants were recruited from the outpatient clinics of Hepatology, Gastroenterology, and Infectious diseases Department, Al-Azhar University Hospitals, Cairo, Egypt. The study conformed to the medical ethics of the 1975 Declaration of Helsinki, and all participants provided written informed consent.

Sample and data collection

A single fecal sample was obtained from each subject and was collected in a dry, clean and leak-proof labeled plastic container. A questionnaire containing demographic, clinical and environmental data was obtained with each sample. The designed questionnaire was modified from Da'as [13] and Mor et al.[14]

Work plan and sample processing

Macroscopic examination of all collected fecal samples was conducted at first regarding physical characteristics, presence of blood, mucus and macroscopic parasite elements. All collected fecal samples were examined coproscopically, then subjected to copro-molecular assays.

Each sample was divided into three portions:

- A small part of each specimen for direct smear examination using a microscope.
- Another part of the specimens was preserved in tight containers using formol-saline 10 % as a fixative for coproscopic examination and staining with acid-fast stain.
- The rest of the specimen was stored at -20ºc in Eppendorf tubes for molecular study.

Coproscopy was carried out in the Diagnostic and Research Unit of Parasitic Diseases (DRUP), Medical Parasitology Department, Kasr Al-Ainy Faculty of Medicine, Cairo University, Egypt. and copro-nPCR assay was held in Lab of Molecular Medical Parasitology (LMMP), Medical Parasitology Department, Kasr Al-Ainy Faculty of Medicine, Cairo University, Egypt.

I-Direct wet smear.[15] Wet smears were examined directly using saline and a drop of iodine was added to another slide.

II. Concentration using modified Ritchie's biphasic method.

This is a modification of formol ethyl-acetate concentration method according to Garcia.[15]

III. Specialized staining of *Cryptosporidium* spp. Oocysts Cold kinyoun's AF stain.

All fecal samples were permanently stained to detect cryptosporidial oocysts using cold kinyoun's AF stain according to Garcia. [15]

IV. Copro-nPCR / RFLP assays.
i. Genomic DNA extraction from stool samples.

This was done using Favor Prep stool DNA isolation Mini Kit (Favorgen Biotech corporation ping-Tung 908, Taiwan, Cat. No. FASTI001) following the manufacturer's instructions.

ii. Copro n-PCR- RFLP assay. [16]
Amplification of the extracted genomic DNA was done using the nPCR assay targeting the *Cryptosporidium* cowp gene. The nPCR was done using the 2 sets of 2 pairs of primers described earlier,[16] where the first set primers were used to produce a DNA template for the nested reaction. The second set of primers was used to anneal internally within the previously obtained amplicon (769 bp) in order to increase the specificity of *Cryptosporidium* detection. PCR products of 2ry PCR (533 bp) were visualized on 1.5% agarose gel after ethidium bromide staining using gel electrophoresis and visualized on a UV transilluminator.

The PCR products of nPCR were digested by RasI restriction enzyme (Thermo Scientific) and the resulting restriction fragments according to manufacture instruction and resolved using 3% Metaphor agarose after ethidium bromide staining.

Statistical Analysis
Data were collected, revised, coded and entered into the Statistical Package for Social Science (IBM SPSS) version 23. The quantitative data were presented as mean, standard deviations and ranges when parametric. Also, qualitative variables were presented as numbers and percentages. The comparison between groups regarding qualitative data was done by using the Chi-square test and/or Fisher exact test when the expected count in any cell found less than 5. The comparison between two groups regarding quantitative data and parametric distribution was done by using an Independent t-test while more than two groups were compared by using the One-Way ANOVA test. The confidence interval was set to 95% and the margin of error accepted was set to 5%.

RESULTS

		Liver cirrhotic group (n.=60)		Control group (n.=60)		Total	P-value
		Copro-nPCR		Copro-nPCR			
		+ve	-ve	+ve	-ve		
AF Faecal Microscopy	+ve	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0)	
	-ve	2 (3.3%)	58 (96.7%)	0 (0.0%)	60 (100%)	60 (100%)	0.154
Total		2 (3.3%)	58 (96.7%)	0 (0.0%)	60 (100%)	60 (100%)	

Table 1: The prevalence of *Cryptosporidium* among liver cirrhotic group and control group using copro-nPCR and AF fecal microscopy.

The previous table showed that the prevalence of cryptosporidial DNA using copro-nPCR was 3.3% (2 cases) among the study group with no statistical significance (*P*-value was > 0.05), none of them were detected by microscopic examination of AF stained fecal smears. None of the control group were positive molecularly or microscopically for Cryptosporidium DNA or oocysts, respectively.

		Study group
		No. = 60
HCV or HBV	HCV	51 (85.0%)
	HBV	9 (15.0%)
HCC	HCC	29 (48.3%)
	No HCC	31 (51.7%)
Child score	Child A	4 (6.7%)
	Child B	18 (30.0%)
	Child C	38 (63.3%)

Table 2: Distribution of cases of viral hepatitis (HCV and HBV), HCC and Child score among the liver cirrhotic group.

The previous table showed that among liver cirrhotic patients, 85% of patients were HCV positive and 15% were HBV positive, 48.3% (29/60) of them were suffering from HCC. The grades of liver cirrhosis were Child A, Child B and Child C 6.7%, 30%, and 63.3% respectively.

		HCV or HBV		*P*-value	Sig.
		HCV	**HBV**		
Cryptosporidium by copro-nPCR	No	49 (96.1%)	9 (100.0%)	0.546	NS
	Yes	2 (3.9%)	0 (0.0%)		

Table 3: Distribution of *Cryptosporidium* detected two cases by copro-nPCR among hepatitis (HCV, HBV) patients of the liver cirrhotic group.

		HCC		P-value
		HCC	**No HCC**	
Cryptosporidium by copro-nPCR	No	27 (93.1%)	31 (100.0%)	0.137
	Yes	2 (6.9%)	0 (0.0%)	

Table 4: Distribution of *Cryptosporidium* detected two cases by copro-nPCR among HCC patients of the liver cirrhotic group.

		Child score			P-value	Sig.
		Child A	**Child B**	**Child C**		
Cryptosporidium by copro-nPCR	No	4 (100.0%)	18 (100.0%)	36 (94.7%)	0.549	NS

Table 5: Distribution of *Cryptosporidium* detected two cases by copro-nPCR among hepatitis (HCV, HBV) patients of the liver cirrhotic group.

The previous tables (Tables 3,4 and 5) showed that the detected two *Cryptosporidium* were among HCV patients, patients with HCC and Child C with no statistical significance (*P*-value was > 0.05).

		Cryptosporidium by copro-nPCR		Test value	P-value	Sig.
		No	**Yes**			
		No. = 58	**No. = 2**			
Gender	Male	32 (55.2%)	1 (50.0%)	0.021	0.885	NS
	Female	26 (44.8%)	1 (50.0%)			
Age	Mean ± SD	46.41 ± 8.06	46.50 ± 24.75	-0.014	0.989	NS
	Range	29 – 58	29 – 64			
Residency	Rural	30 (51.7%)	1 (50.0%)	0.566	0.754	NS
	Semiurban	18 (31.0%)	1 (50.0%)			
	Urban	10 (17.2%)	0 (0.0%)			
Water source	Well/pump	5 (8.6%)	1 (50.0%)	3.724	0.155	NS
	Tap	49 (84.5%	1 (50.0%)			
	Bottled	4 (6.9%)	0 (0.0%)			
	Bottled+tap	0 (0.0%)	0 (0.0%)			
Contact with animals	No	44 (75.9%)	1 (50.0%)	0.690	0.406	NS

Table 6: Demographic and environmental data of copro-nPCR positive *Cryptosporidium* cases among the liver cirrhotic group.

		Cryptosporidium by copro-n PCR		P-value	Sig.
		No	**Yes**		
		No. = 58	**No. = 2**		
Main complaint	Diarrhea	29 (50.0%)	1 (50.0%)	0.126	NS
	Abdominal discomfort	24 (41.4%)	0 (0.0%)		
	Abdominal pain	5 (8.6%)	1 (50.0%)		
Stool content	Normal	55 (94.8%)	2 (100%)	0.947	NS
	Blood	2 (3.4%)	0 (0.0%)		
	Mucus	1 (1.7%)	0 (0.0%)		
	Blood and mucus	0 (0.0%)	0 (0.0%)		
Stool consistency	Formed	27 (46.6%)	1 (50.0%)	0.847	NS

Table 7: Clinical data and macroscopic results of copro-nPCR positive *Cryptosporidium* cases among the liver cirrhotic group. The previous tables (Tables 6 and 7) showed that none of the demographic, environmental or clinical data of positive *Cryptosporidium* cases by copro-nPCR among liver cirrhotic patients showed any significant association with *Cryptosoriudium* infection (P-value > 0.05).

DISCUSSION

Cryptosporidium parvum was detected in liver cirrhotic studied patients with HCC and liver cirrhosis of Child-Pugh class C, but the prevalence was relatively low (3.3%) compared to other studies from Egypt and other countries. An earlier study conducted in Egypt showed a high prevalence of *Cryptosporidium* infection of 30% (45/150), 22% (11/50), 36% (18/50), 32% (16/50) in patients who had chronic liver disease with diarrhea, liver cirrhosis without ascites, liver cirrhosis with ascites and HCC respectively, while a prevalence rate of 14% (7/50) were detected in controls using microscopy and ELISA.[6] In Nepal, *Cryptosporidium* oocysts were detected in the stools of 20% of patients with liver diseases using microscopy.[17] In agreement with our results, many studies reported a similar prevalence rate. A study in China found that the prevalence of *Cryptosporidium* infection in the HBV-associated acute on chronic liver failure patients was 6 % with much lower prevalence rate of 0.8% in patients with chronic hepatitis B.[19] A study conducted at Ahmed Maher Teaching Hospital, Cairo, Egypt, found that the prevalence of *Cryptosporidium* among HBV chronic infection cases was only 2.5% (3/120).[20]

Explanation of our findings and findings of some of the above studies might be that hepatitis itself does not increase susceptibility to cryptosporidiosis but if associated with decompensated progressive liver cirrhosis which caused the impaired cellular immune response in these patients, it makes them more susceptible to several opportunistic infectious agents, including *Cryptosporidium spp*.[5,6]

The only detected *Cryptosporidium* spp. in the present study was the zoonotic type, *Cryptosporidium parvum*. In Egypt, the predominant species in human are *Cryptosporidium hominis* and *Cryptosporidium parvum* with variance in the predominance of one more than the other in different study situations.[21-23]

The *Cryptosporidium* prevalence is widely varied between countries and time. Reports showed that the *Cryptosporidium* prevalence is usually higher when molecular methods were used. In limited-resource countries and developing countries, including Egypt, *Cryptosporidium* infection occurs mostly in children younger than 5 years of age and in AIDS patients.[19,24-27]

In the present study, there was no association between *Cryptosporidium* and demographic, environmental or clinical data. Demographic, Environmental and clinical data could have an important role in the prevalence or risk of exposure of cryptosporidiosis in children, however, the exact mechanisms behind are not clear. These risk factors may affect susceptibility to cryptosporidiosis but do not affect the course of the disease or the disease pathogenicity.[28-30]

The age of patients in our study was above 29 years old, and the mean age was 46 years. The high incidence and prevalence of *Cryptosporidium* previously reported is for children less than 12 years in Egypt as well as worldwide.[26,28-32]

In our study, the mean age of patients was 46 years, an age where it is rare to become infected by *Cryptosporidium,* which may explain the low prevalence of *Cryptosporidium* in our study. Also, differences in the subject selection, as we selected only patients with liver cirrhosis may account for low prevalence. In addition, the study sample size was relatively small due to the availability of complicated liver cirrhotic cases. Other studies used microscopy which is a tool of limitations and leads to false-positive results as it is sometimes difficult to differentiate *Cryptosporidium* from yeasts, in addition to false-negative results due to low sensitivity. Due to the higher sensitivity of PCR-based methods, many authors considered copro-nPCR the gold standard in *Cryptosporidium* detection.[9,26]

CONCLUSION

There was a low prevalence of *Cryptosporidium parvum* among liver cirrhotic Egyptian patients with HCV, suffering from HCC and had grade C liver cirrhosis irrespective of their data. Copro-nPCR is more sensitive than coproscopy for the detection of *Cryptosporidium* infection. Our findings indicated the need for molecular searching of *Cryptosporidium* of populations where hepatitis is associated with decompensated progressive liver cirrhosis to avoid delay in the final diagnosis.

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