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Mohammed Elhussiny

Histology Department, Faculty of Medicine, Al-Azhar University, Cairo, Egypt.,
m.elhussiny.med@gmail.com

Mohamed El-Faramawy

Medical Parasitology Department, Faculty of Medicine, Al-Azhar University, Cairo, Egypt.,
drfarammwympara@gmail.com

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Study of Cryptosporidium Infection in Liver Cirrhosis and Animal Model

Mohammed E.A. Elhussiny,¹ Ph.D., Mohamed Said El-Faramawy,² Ph.D.

* Corresponding Author:

Mohammed E.A. Elhussiny
m.elhussiny.med@gmail.com

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¹Histology Department Department, Faculty of Medicine, Al-Azhar University, Cairo, Egypt.

²Medical Parasitology Department Department, Faculty of Medicine, Al-Azhar University, Cairo, Egypt.

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ABSTRACT

Background: Cryptosporidium is a parasite that infects man especially immunocompromised patients. Liver cirrhosis reduced cellular immunity. Methods were used for diagnosis of cryptosporidium including coproscopy and copro-nested (copro-n) PCR assay.

Aim of the study: Detection of the molecular and genotypic prevalence of Cryptosporidium species in fecal samples of Egyptian patients with cirrhotic liver and exploring the possibility of association between Cryptosporidium and grades of liver cirrhosis, besides assessment their histopathological changes.

Patients and Methods: This cross-sectional study was carried out on 60 patients with chronic liver disease (HCV and HBV positive) classified according to Child-Pugh score and 60 subjects free of any known liver diseases as a control group. Stool samples were taken for coproscopy using acid-fast (AF) stain and copro-nPCR assay. We designed animal model for studying the effect of Cryptosporidium infection by assessing histopathological changes in intestine of immunosuppressed and non-immunosuppressed rats.

Results: There is low prevalence of cryptosporidial infection in patients with liver cirrhosis (3.3%) by using copro-nPCR corresponding to 0% by coproscopy. The prevalence among patients with hepatocellular carcinoma (HCC) and grade C liver cirrhosis were (6.9%) and (5.3%) respectively. The predominant genotype was Cryptosporidium parvum. Regarding animal study, there were no significant intestinal histological changes except a decrease in goblet cells count in immunosuppressed rats.

Conclusion: Copro-nPCR is more powerful than coproscopy for detection of cryptosporidial infection. The prevailing genotype was *C.parvum*. There is no strong association between liver cirrhosis and cryptosporium infection but chance for infection may increase with the development of HCC or when patients become decompensated. Demographic, environmental, and clinical data are not associated with cryptosporidial infection.

Keywords: Cryptosporidium; Liver Cirrhosis; Copro-nPCR, animal study.

In patients having good immunity, diarrhea would be frequently mild and self-limiting. In patients with compromised immunity, though, it can be severe, long-lasting, and life-threatening.⁴

INTRODUCTION

Cryptosporidium is a protozoan parasite which causes diarrhea in humans by infecting epithelial cells in the small intestine. It is a developing zoonosis having reservoirs in cattle, domesticated animals, and fecal polluted environments with a global distribution.¹

Ingestion of *Cryptosporidium* oocysts by polluted food or water (drink and recreation water) or direct human-to-human and animal-to-human contact results in transmission via the fecal-oral pathway. These small-sized oocysts (4-6 µm) are highly resistant to common household disinfectants.²

The risk factors for such protozoans, such as demographic, ecological, and environmental data, reveal that zoonotic transmissions as well as water transmission methods are the main infection routes.³

Previous research has found a link between severe hepatic injury and hepatic failure and a reduction in cellular immunity.⁵ The *Cryptosporidium* was found in 32% of sick people having hepatocellular carcinoma and diarrhea in recent times, comparable to 22% of sick people having liver cirrhosis without ascites and 36% of sick people having liver cirrhosis with ascites. *Cryptosporidium* oocysts have been found in the stools of 20 % of people with hepatic illness.⁶

This type of *Cryptosporidium*-related diarrhea is not uncommon. As a result, laboratory diagnostic tests must be used to make the diagnosis. *Cryptosporidium* infection is not routinely diagnosed in most clinical laboratories, despite significant developments in diagnostic technology.⁷ Few microscopic techniques are routinely employed to detect *Cryptosporidium* in

feces, depending on the oocysts' characteristic morphology. A complicated clinical specimen like feces, on the other hand, makes identification difficult. It would be also time-consuming and tiresome, and it necessitates a significant deal of expertise.⁸

Neither microscopy nor coproantigen assays are capable of achieving this goal. As a result, various PCR-based assays for detecting and characterizing *Cryptosporidium* DNA in feces have been recently developed.⁹

The application of molecular epidemiologic methods has revealed fresh information about the variety of *Cryptosporidium* species that infect animals and humans.¹⁰ There are a total of 26 *Cryptosporidium* species known so far.¹¹ *C.hominis* and *C.parvum* are responsible for the majority of human cases.¹⁰ Many other *Cryptosporidium* species, such as *C. felis*, *C. meleagridis*, *C. cuniculus*, *C. canis*, and *C. ubiquitum*, are found in human beings at a lesser frequency.¹⁰ A new species, *C. viatorum*, has recently been discovered in ten passengers returning from the Indian subcontinent to the United Kingdom.¹²

This study was done for Detection of the molecular and genotyping prevalence of *Cryptosporidium* spp. in stool specimens of Egyptian patients with cirrhotic liver and to investigate the potential for a link between *Cryptosporidium* spp and grades of liver cirrhosis, also to assess their demographic, clinical, and environmental data for risk assessment through a case-control study design. Besides assessing the histopathological changes in rats.

PATIENTS AND METHODS

Study population

This cross-sectional study included 60 patients (33 men and 27 women) with chronic liver disease (HCV and HBV positive) and 60 participants (32 men and 28 women) without any known liver disorders as a control group, who were matched for ethnicity, gender, and age.

Patients from El Hussein University Hospital and Kasr Al-Ainy University Hospital were chosen. Stool samples were collected from September 2017 to January 2020.

Positive anti-HCV and reverse transcription-polymerase chain (PCR-RT) were used to diagnose hepatitis C virus (HCV), while positive hepatitis B surface antigen (HBsAg) and PCR were used to diagnose hepatitis B virus (HBV). A history, clinical examinations, abnormal liver function testing, and abdominal ultrasonography were used to diagnose cirrhosis of the liver.

Using a modified Child-Pugh score, the patients have been further categorized in order to determine the relationship between *Cryptosporidium* infection and the liver disease severity. The Child-Pugh score is made up of 5 clinical parameters that are used to evaluate the diagnosis of chronic liver illnesses based on ascites degree, bilirubin and albumin plasma concentrations, prothrombin time, and encephalopathy degree. The overall points for every item are represented by the Child-Pugh score. Patients are classified into Child-Pugh grades based on the sum of these points. Grade A (well-compensated disease) is defined as a total score of 5 to 6; grade B (severe functional compromise) is defined as a total score of 7 to 9, and grade C is defined as a total score of 10 to 15 (decompensated disease).

Depending on clinical, analytical, and epidemiological data, all known causes of liver diseases other than HCV and HBV were excluded, as were all patients with immunosuppression (such as diabetes and HIV infection) were excluded. In summary, participants were selected from the outpatient clinics of the Departments of Hepatology, Gastroenterology, and Infectious Diseases at Al-Azhar University Hospitals in Cairo). The study followed the 1975 Declaration of Helsinki's medical ethics, and all subjects gave written informed consent.

Sample and data collection.

Each subject provided a single faeces sample, which was collected and labelled in a clean, dry, leak-proof plastic container. With each sample, a questionnaire covering demographic, clinical, and environmental data was acquired. The designed questionnaire was modified from Da'as¹³ and Mor et al..¹⁴

Work plan and sample processing.

All collected fecal samples were examined coproscopically, then subjected for copro-molecular assays.

Each specimen was broken down into 3 portions: A) A small part of each specimen for direct smear examination. B) Another part of the specimens was preserved in tight containers using formalin saline 10 % fixative for coproscopic inspection and staining. C) The remainder of the sample was preserved in eppendorff tubes at -20°C for molecular study.

Coproscopy was performed at Cairo University's Kasr Al-Ainy Faculty of Medicine's Diagnostic and Research Unit of Parasitic Diseases (DRUP). The lab of Molecular Medical Parasitology (LMMP), Medical Parasitology Department, Kasr Al-Ainy Faculty of Medicine, Cairo University, Egypt, conducted the copro-nPCR assay.

Parasitological Coproscopic Examination:

Macroscopic examination of all collected fecal samples was conducted at first regarding physical characteristics, presence of blood, mucus, and macroscopic parasite elements. Then, samples were subjected to wet mounting examination prior to and following formalin-ethyl acetate concentration technique for detection of parasites.

Direct wet smear.

Wet smears were examined directly using saline and a drop of iodine was added to another slide.

Concentration using modified Ritchie's biphasic method.

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

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

All fecal samples were permanently stained to detect *Cryptosporidium* spp. oocyst utilizing cold kinyoun's AF stain according to Garcia.¹⁵

Copro-nPCR / RFLP assays.

Genomic DNA extraction from stool samples.

The stool DNA isolation Mini Kit from Favor Prep was used (Favorgen Biotech corporation ping-Tung 908, Taiwan, Cat. No. FASTI001).

Principle

This is a solid phase spin column method. It involves; cell disruption with digestion of proteins and their degradation under denaturing conditions during 95°C incubation with proteinase K. Buffering conditions were then adjusted to be of a high ionic strength to allow adsorption of DNA onto the silica membrane. Washing away impurities (stays of digested proteins as well as other impurities that could hinder PCR and other downstream enzymatic operations) using a prepared alcohol solution. Under low ionic strength conditions equilibrated to room temp, pure and concentrated DNA was extracted from the Mini spin column.

Amplification of extracted genomic DNA (Copro n-PCR).

Principle

In vitro, PCR is a technique for enzymatically amplifying a particular DNA region which sits between two known DNA sequence areas. It is based on thermal cycling, which involves heating and cooling the reaction repeatedly for DNA melting and enzymatic replication of the DNA with thermostable DNA polymerase (after which the method was named), primer sequence (complementary to the targeted area in the template DNA) and deoxynucleotide triphosphates (dNTPs) (the building units). As PCR advances, the DNA created is

employed as a template for replication, exponentially amplifying the DNA template by up to one billion times.¹⁶

Two sequential sets of primers were employed in two consecutive reactions in extended (nested) PCR. A pair of external primers are utilized to create DNA products in the first PCR.

The products from the first PCR are then used as templates in a second PCR, using two different primers whose binding sites are located (nested) within the first set (are internal to the first primer pair), thus increasing specificity. Nested PCR is often more successful in specifically amplifying long DNA products than conventional PCR.¹⁷

Detection of PCR products

Following ethidium bromide staining, PCR products have been visualized using gel electrophoresis and a UV transilluminator on a 1.5 % agarose gel.¹⁸

RFLP analysis and molecular characterization of the amplified products.

The DNA sample is fragmented and digested with restriction enzymes, with the resultant restriction fragments separated by gel electrophoresis as per their lengths.

After ethidium bromide staining, the amplified products of nPCR of positive samples were digested using restriction enzymes according to the manufacturer's instructions and resolved with 3% Mataphore.

Experimental animals

The animal model for this study was 30 adult healthy albino rats weighing from 150 to 200 grams each. They were kept in suitable cages (30x32x30 cm for every five rats) at room temp and on a natural light/dark cycle. They were fed standard food as well as bread and green vegetables and were given unlimited access to water. Rats have been randomly assigned to one of two groups: control (CNT) and *Cryptosporidium* oocysts inoculated (Cry) group. Using 18–20 gauge feeding tubes, oocysts were inoculated to rats in the Cry group via oral-gastric gavage. Each rat was given 200 µl liters of PBS with various 10⁵ oocysts¹⁹. Cry group were subdivided into two subgroups. Two weeks before *Cryptosporidium* oocysts inoculation, one subgroup (Cry-AZ) was given drinking water that contains azathioprine as immunosuppressant drug (Sigma Aldrich A4638) has been dissolved at 0.04 mg/mL and the estimated dose was 6-20 mg/kg body weight per day, as stated by others before²⁰. The rest of the rats in Cry group did not receive any treatment. The CNT group was given PBS as a control vehicle. Rats were followed up to 50 days. On day 51, all rats were sacrificed.

Histopathology study

Sections of the duodenum were imbedded in a 10% formaldehyde solution for the fixation method, and tissues were treated with graded alcohols, xylene, and paraffin. Afterwards, in a transverse plane, 5- μ m-thick sections of blocked duodenal tissue were produced with a rotary microtome. For examination under light microscopy at 100x, all the sections were placed on glass slides and stained with hematoxylin and eosin (H&E). As Bethune et al. did, we calculated the villus height/crypt depth (V:C) ratios from linear measurements of 5 villus heights divided by the corresponding crypt depths. Villus height was measured linearly from the top of the villi to the mouth of the Lieberkuhn crypt, and crypt depth was measured from the mouth of the Lieberkuhn crypt to the upper border of the intestinal lamina muscularis²¹.

We performed quantitative histological studies for goblet cells. Sections were stained by PAS and H&S then viewed under light microscopy at 400x. The total number of PAS positive cells and number of epithelial cells were counted in a blinded fashion for 15 to 25 longitudinal sections of villi and crypts. We express the number of goblet cells as number of PAS positive cells per 100 as demonstrated by Katz's lab²².

We also evaluate inflammatory cells in longitudinal sections of intestinal villi. The frequency of inflammatory cells infiltrate was assessed as 1 for

mild infiltrate, 2 for moderate infiltrate and 3 for sever infiltrate²³.

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The image analysis was performed using Image J software.

Statistical Analysis

The data was gathered, edited, coded, and entered into IBM SPSS version 23 (Statistical Package for Social Science). When parametric data was used, mean, standard deviations, and ranges were used to present the quantitative data. Qualitative variables were also provided as numbers and percentages. When the predicted count in any cell was < 5, the Chi-square test and/or Fisher exact test were used to compare groups' qualitative data. The Independent t-test was used to compare quantitative data and parametric distribution between two groups, whereas the One-Way ANOVA test was used to compare more than two groups. The margin of error accepted was set to 5%, with a 95% confidence interval.

RESULTS

		Patients group No. = 60
Cryptosporidium by acid fast stain	No	60 (100.0%)
	Yes	0 (0.0%)
Cryptosporidium by PCR	No	58 (96.7%)
	Yes	2 (3.3%)

Table 1: Cryptosporidium by acid-fast stain and cryptosporidium by PCR among the patient's group.

		Patients 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: ABPI in the studied patients' groups pre- and post-operative.

		HCV or HBV		Test value	P-value	Sig.
		HCV	HBV			
Cryptosporidium by PCR	No	49 (96.1%)	9 (100.0%)	0.365	0.546	NS
	Yes	2 (3.9%)	0 (0.0%)			

Table 3: Relation of cryptosporidium detection by PCR to HCV or HBV in patients' group. (P-value > 0.05: Nonsignificant; P-value < 0.05: Significant; P-value < 0.01: Highly significant*: Chi-square test; •: Independent t-test).

		HCC		Test value	P-value	Sig.
		HCC	No HCC			
Cryptosporidium by PCR	No	27 (93.1%)	31 (100.0%)	2.212*	0.137	NS
	Yes	2 (6.9%)	0 (0.0%)			

Table 4: Relation of cryptosporidium detection by PCR to HCC in patients' group. (P-value > 0.05: Nonsignificant; P-value < 0.05: Significant; P-value < 0.01: Highly significant*: Chi-square test; •: Independent t-test).

		Child score			Test value	P-value	Sig.
		Child A	Child B	Child C			
Cryptosporidium by PCR	No	4 (100.0%)	18 (100.0%)	36 (94.7%)	1.198*	0.549	NS
	Yes	0 (0.0%)	0 (0.0%)	2 (5.3%)			

Table 5: Relation of cryptosporidium detection by PCR to Child score in patients' group. (P-value > 0.05: Nonsignificant; P-value < 0.05: Significant; P-value < 0.01: Highly significant *: Chi-square test; •: One Way ANOVA test).

		Cryptosporidium by PCR		Test value	P-value	Sig.
		No No. = 58	Yes 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
	Yes	14 (24.1%)	1 (50.0%)			

Table 6: Relation of the prevalence of cryptosporidium by PCR to demographic and environmental data in patients' group. (P-value > 0.05: Non significant; P-value < 0.05: Significant; P-value < 0.01: Highly significant).

		Cryptosporidium by PCR		Test value	P-value	Sig.
		No No. = 58	Yes No. = 2			
Main complaint	Diarrhea	29 (50.0%)	1 (50.0%)	4.138	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%)	0.109	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%)	0.333	0.847	NS
	Soft	23 (39.7%)	1 (50.0%)			
	Watery	8 (13.8%)	0 (0.0%)			

Table 7: Relation of clinical data, macroscopic results to the prevalence of cryptosporidium by PCR in patients group. (P-value > 0.05: Non significant; P-value < 0.05: Significant; P-value < 0.01: Highly significant).

Cryptosporidium by PCR	Control group No. = 60	Patients group No. = 60	Test value*	P-value	Sig.
No	60 (100.0%)	58 (96.7%)	2.034	0.154	NS
Yes	0 (0.0%)	2 (3.3%)			

Table 8: Comparison between control group and patients' group regarding cryptosporidium by PCR. (P-value > 0.05: Nonsignificant; P-value < 0.05: Significant; P-value < 0.01: Highly significant).

Regarding histological studies in our animal model, we assessed the villus height and crypt depth (V/C) in the duodenum. We found a reduction in V/C ratios in Cry and Cry+AZ groups compared to the CNT group which indicates villus atrophy, but it wasn't statistically significant ($F=2.99$, $P=0.0634$, One way ANOVA) (Figure 1 & 2).

We performed Periodic Acid Schiff (PAS) staining for detection and making quantitative analysis for goblet cells. There was a mild significant decrease in

goblet cells count in Cry+AZ group while in Cry group there was no change ($F=6.23$, $P=0.01$, One way ANOVA, Tukey's multiple comparisons test: CNT vs Cry+AZ $p<0.05$) (Figure 1 & 3).

We studied inflammatory cells infiltrate in intestinal villi. There were some changes between Cry and Cry+AZ and CNT groups but without statistically significance ($F=0.326$, $P=0.73$, One way ANOVA) (Figure 1 & 4).

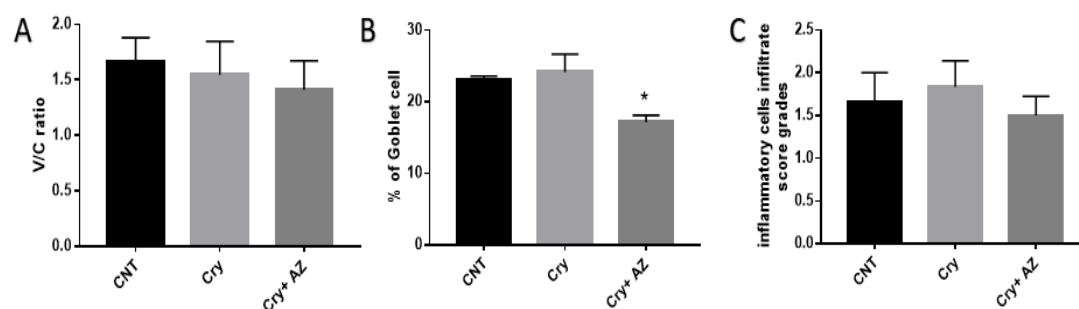


Fig 1: (A) V/C ratio in the duodenum of CNT, Cry and Cry+AZ groups. It shows no significant changes between the groups. (B): It shows goblet cells count in each 100 epithelial cells. There was a significant reduction in goblet cells count in Cry+AZ group. (C) Score grades for inflammatory cells infiltrate in the duodenum of CNT, Cry and Cry+AZ groups. There were no significant changes between the groups.

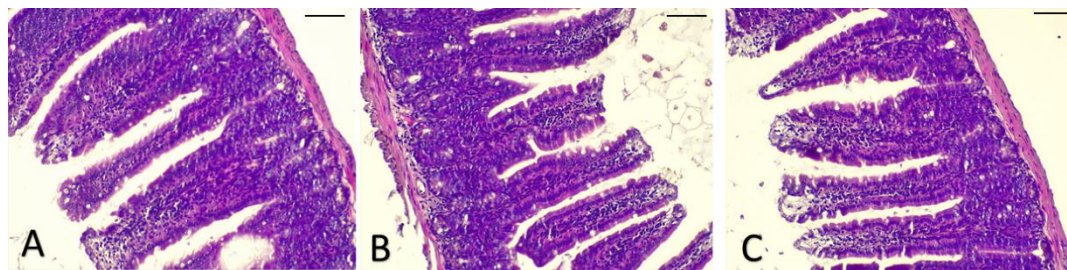


Fig 2: Representative images of H&E-stained duodenum showing normal morphology of the villi in the control group (Image A) with atrophy of some villi in Cry group (Image B) and Cry+AZ group (Image A) (100X, scale bar 100 μ m).

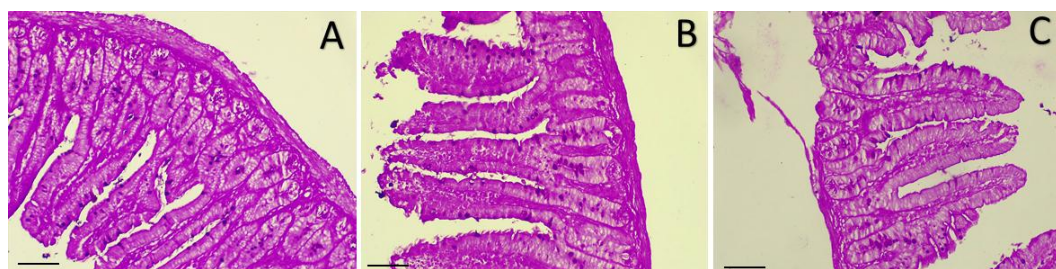


Fig 3: Representative images of PAS-stained goblet cells in the duodenum. There was no statistically significant difference in goblet cells count between Control (Image A) and Cry group (Image B). There was a mild decrease in goblet cells count in Cry+AZ group (Image A) vs control (100X, scale bar 100 μ m).

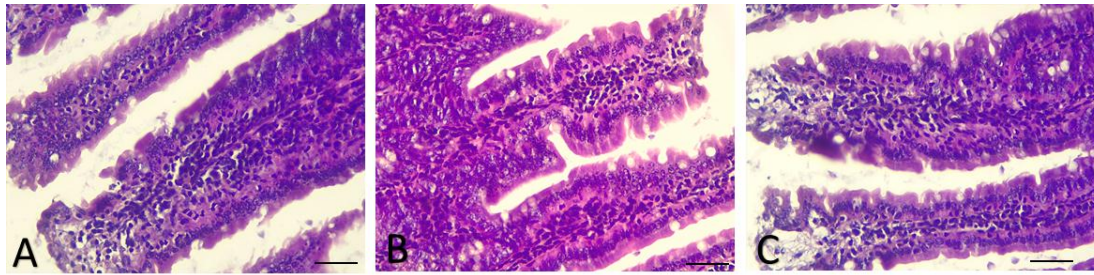


Fig 4: Representative images of H&E-stained duodenum showing inflammatory cellular infiltrate. Control group (Image A); Cry group (Image B) and Cry+AZ group (Image A) (200X, scale bar 20 μ m).

DISCUSSION

The key goal of our study was detection of the molecular and genotyping prevalence of *Cryptosporidium* spp. in fecal samples of Egyptian patients with cirrhotic liver and exploration of the possibility of association between *Cryptosporidium* spp and grades of liver cirrhosis, also assessment of their demographic, clinical and environmental data for risk assessment through a case-control study design.

Cryptosporidium infection was documented in individuals ranging in age from 3 days to 95 years old all over the world.²⁵ In Egypt, the prevalence of *C. parvum* ranged from 0%–47%. *Cryptosporidium* parvum prevalence among Egyptian military recruits was 31.1%.²⁶

Previous research has linked severe liver injuries and liver failure to decreased cellular immunity.⁵ In people with chronic liver disease, decreased cellular immune function may increase their vulnerability to infection, which include infection with *Cryptosporidium*.⁶ *Cryptosporidium* oocysts were found in the faeces of 20% of liver disease patients.²⁷

Regarding the methods of detection, the obtained results of the current research were in accordance with many studies. Similar findings were obtained by Kaushik et al.²⁸ when *Cryptosporidium* oocysts were detected in 4.6% (7), 3.9% (6), and 13.7% (21) (n=153). by AF stain, ELISA, and PCR, respectively in children suffering from diarrhea.

The obtained results were in disagreement with another one in Cairo, Egypt that reported the most sensitive method in detecting the incidence of *Cryptosporidium* infection in diarrhea patients was ICT as the rate was 5.9%, 5.1% or 4.6%, by ICT, microscopy, and PCR respectively. While prior studies have shown that fecal PCR has a high sensitivity rate, the existence of bilirubin, bile salts, as well as other pollutants in feces might hinder DNA extension in PCR reactions, rendering detection less reliable, according to the author.²⁹ Several authors considered PCR to be the gold

standard because of its higher sensitivity.^{9,30-33} In the present study we nominated nPCR assay as our gold standard.

Also, the fact that mixed infections, re-infections, as well as cross-infections with intestinal and gastric *Cryptosporidium* species may occur in human cryptosporidiosis could be an added explanation why we got a higher sensitivity using molecular detection methods than parasitological and immunological ones in our study.³⁴

The most common genotypes in people are *C. hominis* and *C. parvum*.³⁵ In our study revealed only that the genotype was *C. parvum*.

This finding matched those of another study conducted in Egypt, which found that *C. parvum* predominated over *C. Hominis*.³⁶ However, this contradicts the findings of another study in Egypt, which found a higher incidence of *C. hominis* than *C. Parvum*.²⁹

In terms of the lower prevalence 3.3% (2/60) in sufferers having chronic liver cirrhosis and (0%) in the control group, our study agreed with study in Human province, China found that the frequency of cryptosporidium infection was present in 0.8 % (1/122) of chronic HBV infection cases.³⁷ Also another study was done in Ahmed Maher Teaching Hospital, Cairo, Egypt found that the prevalence of *cryptosporidium* among HBV chronic infection cases was only 2.5% (3/120).³⁸

Our study was in disagreement with another study done in Egypt, which showed that the incidence of *Cryptosporidium* infection in sufferers having chronic liver diseases was 30% (45/150) compared to 14% (7/50) in controls.⁶

Regarding hepatocellular carcinoma, grades of liver cirrhosis to the prevalence of *cryptosporidium*, we found that only two patients who harbored *Cryptosporidium* had hepatocellular carcinoma (6.9%) and were Child C (5.3%) compared to (0%) in patients without HCC or in patients with Child A or B, so study showed that *cryptosporidium* infection increased with development of hepatocellular carcinoma and chronic liver disease progressed from

Child-Pugh class A to Child-Pugh class C but the prevalence was low.

This finding could be explained by the fact that people with liver cirrhosis have a weakened cellular immune response.⁵ In a recent study in Egypt, *Cryptosporidium* was discovered in 32 % of people suffering hepatocellular carcinoma and diarrhea, and 36 % of people suffering liver cirrhosis and ascites.⁶

Many studies have been conducted to investigate the epidemiology and diagnosis of *Cryptosporidium* infection in malignancy patients. Indian research of 560 individuals with cancer and diarrhea discovered a low frequency of *Cryptosporidium*; oocysts were detected within just seven subjects (1.3 %). Five of the seven individuals were diagnosed with hematological cancer.³⁹

Environmental, clinical, and host behavioral factors including gender, age, symptomatic/asymptomatic, drinking water and interaction with animals having feces consistency may have an important role in the risk of cryptosporidiosis exposure or incidence.

These factors may affect susceptibility to infection but have no effect on pathogenicity or disease progression. However, the exact mechanisms behind the increased risk of illness in children are not yet fully clear.⁴⁰⁻⁴¹

Regarding gender, we found no association between gender and the prevalence of *cryptosporidium*.

The age of the host is a major factor in epidemiology of cryptosporidiosis. Despite the fact that cryptosporidiosis impacts people of all ages, monitoring data revealed that children under the age of 12 years old had the highest number of recorded cases. In Canada, New Zealand, the United States, England and Wales, as well as France, there has been a high prevalence of the disease in this age range.⁴² According to Abdel-Messih et al.⁴³, children under the age of 12 are responsible for 61.9 % of instances in Egypt; however, there are no evident discrepancies between the ages of 2 and 5, as earlier observed for Egypt.

The type of water supply was reported by all study participants. However, none of the drinking water sources in our research were substantially related to the probability of developing cryptosporidiosis. This may be attributed to that most of study individuals (both symptomatic and asymptomatic) depend mainly on tap and bottled water as the main source of water supply. Water supplies are a well-known risk factor for cryptosporidiosis.⁴⁴ Al-Warid et al.⁴⁵ in the North of Baghdad confirmed that there was a significant relation between water source and the prevalence of *Cryptosporidium*.

The minimum infectivity rate was observed in patient who drunk boiled water, while the maximum level was recorded among the patients drinking tap

water. These results were not very different from those reported by *Beach*⁴⁶ and *Garvey and McKeown*⁴⁷ in a study in Ireland.

Regarding to contact with animals, we found no association between it and prevalence of *cryptosporidium*.

Nevertheless, Iqbal et al.⁴⁸ suggests contact with infected animals as a possible mode of infection transmission. However, there have been several studies that have failed to find an animal contact association.^{3,49,50}

Regarding complaint and macroscopic examination, there was no significance between prevalence of *cryptosporidium* and these elements.

Many studies have found a link between *Cryptosporidial* infection and diarrhea, abdominal pain, flatulence, itch, vomit, or appetites loss.^{51,52}

We hypothesized diarrhea that is presented in some patients may be related to pathological changes in the intestine which may be adding as a tool for diagnosis of *Cryptosporidial* infection. It was difficult for making this part of the study in patients, so we designed animal models of *Cryptosporidial* infection, and we studied also the effect induced by Azathioprine as immunosuppressant drug in infected models. We found intestinal atrophy in infected groups with and without Azathioprine treatment but did not reach to be significant when compared with control. It may be related to the duration of infection and Azathioprine treatment in our animal models. Also, diarrhea that occurred in some patients may be related to other factors.

These results were matching with previous study that explained *Cryptosporidium* infection either in immunocompetent and immunocompromised patients induces a limited degree of apoptosis in intestinal epithelium as the parasite in the early stage of infection depends on the host cell for growth and development.⁵³

Goblet cells secrete mucus which is composed of mucus glycoproteins that form a viscous film at intestinal epithelial surface. One of its main functions is to maintain the normal commensal bacteria in the gut lumen besides its role in intestinal barrier function. We found a significant decrease in intestinal goblet cells counts in immunosuppressed rats while in the other group there was no change. This result may reflect the intestinal injury induced by *Cryptosporidium* infection and immunosuppression. Regarding inflammatory cells infiltration, we didn't find significant changes between groups.

Our results were in agree with another study that found an obvious decrease in goblet cells count in immunosuppressed animals.⁵⁴

CONCLUSION

Copro-nPCR is more powerful than coproscopy for detection of cryptosporidial infection. The prevailing genotype was *C.parvum*. There is no strong association between liver cirrhosis and cryptosporidial infection but chance for infection may increase with the development of HCC or when patients become decompensated. Demographic, environmental, and clinical data are not associated with cryptosporidial infection. Diarrhea that is presented in some cryptosporidial infected and immunocompromised patients is related to change in intestinal mucus production which reflect the decrease in goblet cells count

REFERENCES

- Davies AP and Chalmers RM. Cryptosporidiosis. *BMJ*. 2009; 339:4168.
- Surl CG, Jung BD, Park BK, et al. Resistance of *Cryptosporidium parvum* oocysts following commercial bleach treatment. *Korean J Vet Res*. 2011; 51: 101-5.
- Youssef FG, Adib I, Riddle MS, et al. Review of Cryptosporidiosis in Egypt. *J Egypt Soc Parasitol*. 2008; 38(1):9-28.
- Arora DR and Arora B. AIDS-associated parasitic diarrhoea. *Indian J Med Microbiol*. 2009; 27: 185-90.
- Wasmuth HE, Kunz D, Yagmur E, et al. Patients with acute or chronic liver failure display 'sepsis-like' immune paralysis. *J Hepatol*. 2005; 42: 195-201.
- Mousa N, Abdel-Razik A, El-Nahas H, et al. Cryptosporidiosis in patients with diarrhea and chronic liver diseases. *J Infect Dev Ctries*. 2014; 8(12):1584-90.
- Desai NT, Sarkar R and Kang G. Cryptosporidiosis: an under-recognized public health problem. *Trop Parasitol*. 2012; 2: 91-8.
- McHardy IH, Wu M, Cohen RS, et al. Clinical laboratory diagnosis of intestinal protozoa. *J ClinMicrobiol*. 2013; (doi: 10.1128/JCM 02877-13).
- Jex AR, Smith HV, Monis PT, et al. Cryptosporidium-biotechnological advances in the detection, diagnosis and analysis of genetic variation. *Biotechnol Adv*. 2008; 26: 304-17.
- Xiao L. Molecular epidemiology of cryptosporidiosis: an update. *ExpParasitol*. 2010; 124: 80-9.
- Fayer R and Santín M. Cryptosporidium xiaoi n. sp. (Apicomplexa: Cryptosporidiidae) in sheep (Ovisaries). *Vet Parasitol*. 2009; 164: 192-200.
- Elwin K, Chalmers RM, Hadfield SJ, et al. Serological responses to *Cryptosporidium* in human populations living in areas reporting high and low incidences of symptomatic cryptosporidiosis. *ClinMicrobiol Infect*. 2007; 13: 1179-85.
- Da'as HA. Prevalence of *Cryptosporidium* species among children ≤ 5 years old in Northwest-Bank, Palestine/ cross sectional study. *Thesis for master's degree in public health, Faculty of Graduate studies, An-Najah National University, Nablus-Palestine*. 2010.
- Mor SM, Tumwine JK, Ndezi G, et al. Respiratory cryptosporidiosis in HIV seronegative children in Uganda: potential for respiratory transmission. *Clin Infect Dis*. 2010; 50(10):13661-72.
- Garcia LS. Diagnostic Medical Parasitology, 5th edition. Washington: ASM Press. 2007; 1202 .
- Cheng S, Fockler C, Barnes, et al. Effective amplification of long targets from cloned inserts and human genomic DNA. *Proc Natl Acad Sci*. 1994; 91(12):5695-9.
- Chou Q, Russell M, Birch DE, et al. Prevention of pre-PCR mis-priming and primer dimerization improves low- copy-number amplifications. *Nucleic Acids Res*. 1992; 20 (7): 1717-23.
- Smith DR. Agarose gel electrophoresis. Methods in molecular biology. In: Murphy, D. and Carter, D.A. Eds. : *Transgenesis Techniques*. Totowa. Humana Press Inc.1993.
- Benamrouz S, Guyot K, Gazzola S, et al. Cryptosporidium parvum infection in SCID mice infected with only one oocyst: qPCR assessment of parasite replication in tissues and development of digestive cancer. *PLoS One*. 2012; 7(12):51232.
- Wielenga MC, de Jeude JF, Rosekrans SL, et al. Azathioprine does not reduce adenoma formation in a mouse model of sporadic intestinal tumorigenesis. *World Journal of Gastroenterology: WJG*. 2014; 20(44):16683.
- Bethune MT, Borda JT, Ribka E, et al. A non-human primate model for gluten sensitivity. *PLoS One*. 2008; 3(2):1614.
- Katz JP, Perreault N, Goldstein BG, et al. The zinc-finger transcription factor Klf4 is required for terminal differentiation of goblet cells in the colon. *Development*. 2002; 129:2619-28.
- Erben U, Loddenkemper C, Doerfel K, et al. A guide to histomorphological evaluation of intestinal inflammation in mouse models. *International journal of clinical and experimental pathology*. 2014; 7(8):4557.
- Neri B, Mossa M, Scucchi L, et al. Histological scores in inflammatory bowel disease. *Journal of Digestive Diseases*. 2021; 22(1):9-22.
- Tzipori S and Ward H. Cryptosporidiosis: biology, pathogenesis and disease. *Microbes Infect*. 2002;4(10):1047-58.
- Badawy A, Gneidy M and Ghoniemy A. Acute diarrhea among military recruits. *Egypt Soc Parasitol*. 2012; 42: 309-20.
- Shrestha S, Larsson S, Serchand J, et al. Bacterial and cryptosporidial infection as the cause of chronic diarrhea in patients with liver disease in Nepal. *Trop Gastroenterol*. 1993; 14: 55-8.

28. Kaushik K, Khurana S, Wanchu A, et al. Evaluation of staining techniques, antigen detection and nested PCR for the diagnosis of cryptosporidiosis in HIV seropositive and seronegative patients. *Acta Trop.* 2008; 107(1):1-7.
29. Abd El-Kader MN, Blanco M, Tammam MA, et al. Detection of *Cryptosporidium parvum* and *Cryptosporidium hominis* in human patients in Cairo, Egypt. *Parasitol Res.* 2011; 110(1):2465-70.
30. Tumwine JK, Kekitiinwa A, Nabukeera N, et al. *Cryptosporidium parvum* in children with diarrhea in Mulago Hospital, Kampala, Uganda. *Am J Trop Med Hyg.* 2003; 68(6): 710-15.
31. El-Hamshary EM, El-Sayed HF, Hussein EM, et al. Comparison of Polymerase Chain Reaction, Immunochromatographic Assay and Staining Techniques in Diagnosis of Cryptosporidiosis. *PUIJ.* 2008; 1(2):77-86.
32. El-Badry AA, Al-Ali KhH and Mahrous AS. Molecular identification & prevalence of *Giardia lamblia* & *Cryptosporidium* in duodenal aspirate in Al-Madinah. *Journal of Medicine and Biomedical Sciences.* 2010; 1(2):47- 52.
33. Salyer S, Gillespie T, Rwego I, et al. Epidemiology and molecular relationships of *Cryptosporidium* spp. in people, primates, and livestock from Western Uganda. *PLoS.* 2012; 6 (4):1-6.
34. Cama VA, Bern C, Roberts J, et al. *Cryptosporidium* species and subtypes and clinical manifestation in children, Peru. *Emerg Infect Dis.* 2008; 14(10):1567-74.
35. Feng Y, Dearen T, Cama V, et al. 90-kilodalton heat shock protein, Hsp90, as a target for genotyping *Cryptosporidium* spp. known to infect humans. *Eukaryot cell.* 2009; 8(4):478-82.
36. Eida AM, Eida MM and El-Desoky A. Pathological studies of different genotypes of human *Cryptosporidium* Egyptian isolates in experimentally mice. *J Egypt Soc Parasitol.* 2009; 39: 975-90.
37. Yu Z, Li F, Zeng Z, et al. Prevalence and clinical significance of *Cryptosporidium* infection in patients with hepatitis B virus-associated acute-on-chronic liver failure. *International Journal of Infectious Diseases.* 2011; 15(12):845-8.
38. Ramadan ME, Ramadan ME and Yousef MS. *Cryptosporidium* infection in patients with acute on chronic liver failure (ACLF). *Pluralidade.* 2015; 2(4): 102-15.
39. Tanyuksel M, Gun H and Doganci L. Prevalence of *Cryptosporidium* spp. in patients with neoplasia and diarrhea. *Scand J Infect Dis.* 1995; 27: 69-70.
40. Yoder JS and Beach MJ. *Cryptosporidium* surveillance and risk factors in the United States. *Exp Parasitol.* 2010; 124(1):31-9.
41. Robertson B, Sinclair MI, Forbes AB, et al. Case-control studies of sporadic cryptosporidiosis in Melbourne and Adelaide, Australia. *Epidemiol Infect.* 2002; 128(3): 419-31.
42. Derouin F, Dutoit E and de Monbrison F. Laboratory-based surveillance for *Cryptosporidium* in France, 2006–2009. *Euro Surveill.* 2010; 15(33):19642.
43. Abdel-Messih IA, Wierzb TF, Abu-Elyazeed R., et al. Diarrhea associated with *Cryptosporidium parvum* among young children of the Nile River Delta in Egypt. *J Trop Pediatr.* 2005; 51(3):154-9.
44. Goh S, Reacher M, Casemore DP, et al. Sporadic cryptosporidiosis, North Cumbria, England, 1996-2000. *Emerg Infect Dis.* 2004; 10(6):1007-15.
45. Al-Warid HS, AL-Saqur IM and Mahmood SH. Occurrence of *Cryptosporidium* spp. among People Live in North of Baghdad. *European Journal of Scientific Research.* 2012; 78(4):539-45.
46. Beach MJ. Waterborne: Recreational Water in *Cryptosporidium* and cryptosporidiosis. 2nd edition. USA: Taylors & Francis Group. 2008; 355-61.
47. Garvey P and McKeown P. Epidemiology of human cryptosporidiosis in Ireland 2004-2006: analysis of national notification data. *Eurosurveillance.* 2009; 14 (8):1-5.
48. Iqbal J, Khalid N and Hira PR. Cryptosporidiosis in Kuwaiti children: association of clinical characteristics with *Cryptosporidium* species and subtypes. *J Med Microbiol.* 2011; 60(5): 647-52.
49. Khashba A, Hilali M and El-Hennawis MM. Cryptosporidiosis among children suffering from diarrhea in Banha, Egypt. *J Egypt Soc Parasitol.* 1989; 19 (3):701-5.
50. Mikhail IA, Hyams KC, Podgore JK, et al. Microbiologic and clinical study of acute diarrhea in children in Aswan, Egypt. *Scand J Infect Dis.* 1989; 21(1):59-65.
51. Chauret C, Springthorpe S and Sattar S. Fate of *Cryptosporidium* oocysts, *Giardia* cysts, and microbial indicators during wastewater treatment and anaerobic sludge digestion. *Can J Microbiol.* 1999; 45(3):257-62.
52. Miller WA, Lewis DJ, Pereira MD, et al. Farm factors associated with reducing *Cryptosporidium* loading in storm runoff from dairies. *J Environ Qual.* 2008; 37(5): 1875-82.
53. McCole DF, Eckmann L, Laurent F, et al. Intestinal epithelial cell apoptosis following *Cryptosporidium parvum* infection. *Infection and immunity.* 2000;68(3):1710-3.
54. Chen S, Wang J, Fang Q, et al. Polysaccharide from natural *Cordyceps sinensis* ameliorated intestinal injury and enhanced antioxidant activity in immunosuppressed mice. *Food Hydrocolloids.* 2019; 89:661-7.