CRYPTOSPORIDIUM INFECTION IN PATIENTS WITH ACUTE ON CHRONIC LIVER FAILURE (ACLF)

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Abstract: Acute on chronic liver failure (ACLF) patients had reduced cellular immunity could contribute to an increased susceptibility to infection in subjects with ACLF, including infection with *Cryptosporidium*. This study was carried out to investigate the prevalence and clinical significance of *Cryptosporidium* infection in patients with hepatitis B virus (HBV)-associated ACLF in Ahmed Maher Teaching Hospital, Cairo, Egypt. Fecal samples from patients with HBV-associated ACLF, chronic hepatitis B (CHB) and children with diarrhea were collected; Modified acid fast Ziehl-Neelsen staining (MZN), coproantigen ELISA were used. The prevalence of *Cryptosporidium* infection in the HBV-associated ACLF patients was 9.5%, although was 2.5% and 5% in CHB and children with diarrhea respectively. No watery diarrhea present in *Cryptosporidium*-positive ACLF patients. Among all 220 ACLF patients, 60 had diarrhea, ten of them were *Cryptosporidium*-positive, which is significantly higher than the *Cryptosporidium*-positive rate in patients without diarrhea.

Key words: Cryptosporidiosis, Chronic Hepatitis B (CHB), Acute on Chronic Liver Failure (ACLF), Modified acid fast Ziehl-Neelsen staining (MZN), Coproantigen ELISA.

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1 Introduction & Background

Cryptosporidiosis is a parasitic disease caused by *Cryptosporidium spp.*, These are enteric protozoan parasite with worldwide distribution which may inhabit the gastrointestinal tract of a wide variety of animals including humans [33]. The infectious stages of the parasite (oocysts) are shed in the feaces of infected individuals; they survive in adverse environmental conditions and are spread by direct contact or through contaminants (food and water) [16].

In immunocompetent individuals, *Cryptosporidium* causes acute diarrhea, usually self-limited, nausea, vomiting, loss of appetite, weight loss and fever [2] [15]. Mostly affecting people with immunodeficiency and, in some cases, can be deadly, and is an important cause of morbidity and mortality in immunosuppressed individuals [20].

Cryptosporidium infection has been reported in various populations with severe impairments of immune status, such as those with acquired immune deficiency syndrome (AIDS) [17] [23] acute leukemia [26] and other hematological disorders, [10] interferon deficiencies, [27] graft transplants, [1] and malnourished children [18]. In a study of human immunodeficiency virus (HIV) infected patients, over 37.3% of AIDS patients with diarrhea were found to have a *Cryptosporidium* infection, which often worsened the prognosis of the disease [6].

Chronic hepatitis B virus (HBV) infection affects over 240 million people worldwide and about a half million people die every year due to the acute or chronic consequences leading to liver failure and liver cancer [32].

Acute-on-chronic liver failure (ACLF) is an increasingly recognized entity encompassing an acute deterioration of liver function in patients with cirrhosis, either secondary to superimposed liver injury or due to extrahepatic precipitating factors such as infection culminating in the end-organ dysfunction. A characteristic feature of ACLF is its rapid progression, the requirement for multiple organ supports and a high incidence of short and medium term mortality of 50-90% [14]. Abundant evidence has indicated that the immune status is impaired in patients with ACLF, who are often highly susceptible to microbial infection [29] [36] cite[18].

The purpose of this study was to determine the prevalence and potentially clinical significance of *Cryptosporidium* infection in patients with hepatitis B virus HBV-associated ACLF.

2 Materials and Methods

2.1 Ethical declaration

Ethical approval was obtained from the Committee of Research, Publications and Ethics of Ahmed Maher Teaching hospital, Cairo, Egypt. Informed consent was obtained from each patient who voluntarily participated after a clear explanation of the research objectives. Parents or guardians signed consent on behalf of the children enrolled in this study. The inclusion criteria of participation was: cases of ACLF associated with hepatitis B and chronic hepatitis B (CHB) regardless of age, gender, whereas the exclusion criteria were cases who did not give consent to participate in the study, in addition to Individuals with concurrent hepatitis C virus (HCV), hepatitis E virus (HEV), hepatitis A virus (HAV), or HIV infections or who had autoimmune liver diseases were excluded or children with diarrhea who their ages are more than 2 years old. Clinical data were obtained from patient's medical record with patient's consent and permission from the hospital.

2.2 Subjects

This study was conducted between September 2012 and November 2014. It was carried out on three groups, attending outpatient clinics of Ahmed Maher Teaching Hospital, Cairo, Egypt.

- GROUP 1: 220 patients with acute-on-chronic liver failure (ACLF);
- GROUP 2: 120 patients with chronic hepatitis B (CHB); and
- GROUP 3: 140 children aged less than 2 years with diarrhea.

2.3 Clinical examination

It was done to all adult patients, patients with HBV-associated ACLF and CHB were diagnosed according to previously described criteria by Zhang *et al.* [35]. Late-stage ACLF was defined in the presence of more than grade II hepatic encephalopathy and/or other complications (such as severe hemorrhage, ascites, etc ...).

Recovery in patients with ACLF was defined as [34]:

1. Liver function with normal alanine aminotransferase (ALT) and total bilirubin (TBIL) for 6 months; and
2. Disappearance of clinical manifestations of liver failure after routine treatment for 6 months.

Patients with ACLF other than those who had recovered or died were defined as intractable cases. All patients with ACLF and CHB were followed for 6 months, and fecal samples were collected on days 10, 20, and 30 after the diagnosis of ACLF or CHB; additional fecal samples were collected if diarrhea occurred.

Diarrhea was defined as three or more unformed stools in a 24 hours period. A patient was considered to be infected with *Cryptosporidium* if it was detected positively by Modified acid fast Ziehl-Neelsen staining (MZN) and or coproantigen detection by ELISA.

2.4 Stool specimens and processing

Fresh fecal samples were collected from all patients, into a dry, clean, wide mouth plastic container containing no preservatives, with tight fitting lids. Macroscopic examination was conducted at first regarding several aspects: consistency, presence of blood and mucus and macroscopic parasitic elements. Microscopic examination was then done using direct wet smear technique for the presence of cysts of parasitic protozoa.

All samples were subjected to formalin-ether concentration (centrifugation at 500 g for 10 min) and examined as wet saline and iodine preparation for the detection of parasites. A 0.01 ml sediment sample was thinly spread on two glass slides and air dried. Two dried smears were prepared from fecal concentrate for special staining of *Cryptosporidium* spp. Slides were immediately fixed in methanol for 5 min. [12] A portion of unconcentrated stool samples was stored at -20°C without any preservative for antigen detection.

2.5 Modified Acid Fast Ziehl-Neelsen Staining (MZN) [25] [3]

Is classically performed by staining a methanol fixed thin smear of fecal material with undiluted carbol-fuchsine solution for at least 15 minutes.

Subsequently, the slide is rinsed in tap water and placed in an acid-alcohol solution to remove the stain, while acid-fast structures will resist to the acid-alcohol's destaining action. After rinsing again, the slide is placed for a short period of time in a counter-staining product, such as methylene blue, providing contrast between background material and acid-fast structures.

The slide is rinsed once more and after the slide has been air-dried, The slides were screened under 10X, 40X and 100X of light microscope for identification of *Cryptosporidium*. *Cryptosporidium* oocysts will appear as pink stained, round to oval structures of about 3 to 6 μm in diameter, containing distinct internal structures. The intensity of infection was estimated semi-quantitatively according to the average number of oocysts at 1000 magnification, and the categories established were: negative (absence of oocysts), slight (1-5 oocysts), moderate (6-10 oocysts), and severe ($>$ 10 oocysts) [19].

3 *Cryptosporidium* Antigen detection

It was detected by using a commercial RIDASCREEN®, R-Biopharm AG, Germany. *Cryptosporidium* coproantigen detection is an enzyme immunoassay for the qualitative determination of *Cryptosporidium parvum* in stool samples. This *Cryptosporidium* antigen ELISA described here is of high sensitivity 100%, negative predictive value (NPV) 100.0% [5] and requires no special personnel trained in parasitology to perform it, is quick and simple to use and does not rely on the presence of intact organisms in the stool sample.

The samples were centrifuged at 5,000 rpm (approx. 2,300 g - 2,500 g) for 5 minutes as the test procedure is carried out in an automated ELISA system. High positive patient samples may cause the substrate to precipitate as a black sediment. Samples are considered positive if their extinction is more than 10% above the calculated cut off. Samples are considered equivocal and must be repeated if their extinction is within $\pm$10% of the cut-off.

4 Statistical Analysis

The results of the study were analyzed using SPSS software v. 11.0. The Chi-square test was used to determine the relationships. The Student's t-test was used to compare groups with continuous data measurement.

Table 1: Age and sex distribution among the studied groups

Studied group	**Number of Cases**	**Age range [years]**	**Male**	**Female**
ACLF	220	25-50	25	195
CHB	120	30-50	13	107
Infants with Diarrhea	140	0.3-2	32	108

Cases detected by coproantigen ELISA for *Cryptosporidium* were including the cases detected by MZN. The Frequency of *Cryptosporidium* infection in patients with HBV-associated ACLF was 9.5% (21/220), in CHB patients was 2.5% (3/120), and in children with diarrhea was 5.0% (7/140) (Table 3). Results showed that the rate of positive *Cryptosporidium* infection in HBV-associated ACLF patients was significantly higher than that in CHB patients and that in diarrheic children ($p < 0.01$ for both). ACLF patients with *Cryptosporidium* infection showed no severe or watery diarrhea.

Table 2: Some liver functions tests in the studied groups

Studied Group	**Alanine Aminotransferase (ALT) [U/L]**		**Prothrombin Activity (PTA) [%]**		**Total Bilirubin (TBIL) [μmol/l]**	
	Range	**Median**	**Range**	**Median**	**Range**	**Median**
ACLF	50-13,350	320	22.0-36.5	33	180.0-765.5	353.0
CHB	90-1,890	650	72.0-123.0	92	10.5-36.3	25.8
Infants with Diarrhea	7-34	20	93.0-120.0	102	7.0-34.0	9.0

Table 3: Detection of *Cryptosporidium* infection by Modified acid fast Ziehl-Neelsen (MZN) staining and coproantigen detection in stool

Studied Group	**Patients [un]**	**MZN Stain positive (+ve)**		**Coproantigen Detection positive (+ve)**	
		[un]	**[%]**	**[un]**	**[%]**
ACLF	220	15	6.8	21	9.5
CHB	120	0	0.0	3	2.5
Infants with Diarrhea	140	5	3.5	7	5.0

No significant relation, $p > 0.05$

Table 4: Prevalence of *Cryptosporidium* in late and non late stage of liver failure

ACLF	**Patients**	**Cryptosporidium**			
		positive (+ve)		**negative (-ve)**	
	[un]	**[un]**	**[%]**	**[un]**	**[%]**
Late-Stage Liver Failure	88	7	7.9	81	92.1
Non Late-Stage Liver Failure	132	6	4.5	126	95.5

All positive samples detected in ACLF and CHB patients by MZN (n = 15) or coproantigen detection (n = 21) only showed mild to moderate infection (1-5 oocysts/field of magnification), moderate (6-10 oocysts). However, one diarrheic child had a severe infection (> 10 oocysts/field of magnification).

Clinical characteristics of *Cryptosporidium* infection in patients with ACLF (Table 4), of the 220 patients with HBV-associated ACLF, 88 cases were defined as late-stage liver failure. The rate of positive *Cryptosporidium* infection in patients with late-stage liver failure (7.9%, 7/88) showed no significant difference to that in patients not in the late stage (4.5%, 6/132) ($p > 0.05$).

Of the 88 patients with late stage ACLF (Table 4), 25 cases died or had intractable disease and 63 cases recovered. The rate of positive *Cryptosporidium* infection in the recovered ACLF patients with late-stage liver failure (7.9%, 5/63) showed no significant difference compared with that in patients without recovery (8.0%, 2/25) ($p > 0.05$), suggesting that the severity of HBV-associated ACLF was not associated with *Cryptosporidium* infection in this study.

In addition, the *Cryptosporidium*-positive rate in ACLF patients complicated with infection (9%, 12/133) was significantly higher compared with that in patients without signs or symptoms of infection (2.3%, 2/87) ($p < 0.01$), suggesting that *Cryptosporidium* caused infection in ACLF patients.

Among all 220 ACLF patients, 60 had diarrhea, ten of them were *Cryptosporidium*-positive (16.6%, 10/60), which is significantly higher than the *Cryptosporidium*-positive rate in patients without diarrhea (3.1%, 5/160) ($p < 0.01$). In addition, none of the 20 CHB (n=120) patients with diarrhea

were *Cryptosporidium*-positive. So this study data strongly suggest that *Cryptosporidium* infection may be the cause of diarrhea in patients with HBV-associated ACLF.

5 Discussion

Cryptosporidium parvum is an opportunistic parasite capable of causing gastrointestinal illness in both immunocompetent and immunocompromised patients as well as in children particularly in developing countries [21]. It is now well known that immunosuppressed individuals are at higher risk for *Cryptosporidium* infections, and that carriage of the parasite is associated with diarrheal disease in most cases. Furthermore, in compromised individuals with diarrhea, the disease is much more severe and prolonged than in otherwise healthy individuals [29].

Despite the consensus of opinion regarding the seriousness of *Cryptosporidium* infection in immunocompromised patients and the importance of protecting such patients from infection, there are insufficient valid data to allow an accurate assessment of the prevalence of this infection in this group [13]. Gastrointestinal infections and symptoms of diarrhea often occur in patients with HBV-associated ACLF, however whether *Cryptosporidium* infection is involved in these cases remains unknown [33].

Screening of stool sediment smears by MZN, Modified Ziehl-Neelsen staining is a sensitive and specific approach for the identification of *Cryptosporidium* oocysts in stools [11]. In the present study, MZN stain was at the end in ranking as compared to ELISA In spite of low cost, more time in preparation, staining, reading and interpretation of the smear. Additionally, it requires considerable expertise to identify the oocysts and not to confuse it with other ingredients of stool like yeast spores. In addition, it is not enabling to bulk processing which in accord with [7] and [9] however Weitzel *et al.* [30], reported that coproantigen assays were less time-consuming and easier to perform, but were less sensitive than conventional microscopical methods reported by evaluation of seven commercial antigen detection tests for *Cryptosporidium* in stool samples.

A positive rate of 5% was determined for *Cryptosporidium* infection in children with diarrhea aged younger than 2 years, this is consistent with initial testing of stool samples in a study done in Egypt, 2012 by El-Mohammady *et al.* [8] . These results were not consistent with the findings in other Chinese

reports (0.82-3.90%) [34] [4] [28]. Others reported a high percentage 41% of the studied children less than 1 year old have *Cryptosporidium* in a research done by Sánchez-Vega *et al.* in Mexico in 2006 [24].

Previous studies demonstrated that severe liver injury and liver failure are closely associated with reduced cellular immunity [22]. Reduced cellular immune function could contribute to an increased susceptibility to infection in subjects with chronic liver diseases, including infection with *Cryptosporidium* [35].

Many reports have indicated that patients with ACLF and CHB have different immune statuses [29] [31] which may explain the significant difference in the prevalence of *Cryptosporidium* infection between these two groups of patients.

Clinical findings in this study indicated that the rate of positive *Cryptosporidium* infection in the recovered ACLF patients showed no marked difference to the rate in patients who did not recover. So no marked difference between those with late-stage disease and those with early, or middle, stage disease, this suggested by Yu *et al.* [34].

In AIDS patients, *Cryptosporidium* infection can promote disease progression and worsen the prognosis of the disease [6]. Our investigation showed *Cryptosporidium* infection was not correlated with the progression or severity of ACLF. Nevertheless, *Cryptosporidium* infection occurred in ACLF patients with signs or symptoms of infection. Furthermore, Among all 220 ACLF patients, 60 had diarrhea and ten of them were *Cryptosporidium*-positive (13.8%, 10/60), which is significantly higher than the *Cryptosporidium*-positive rate in patients without diarrhea (3.1%, 5/160) ($p < 0.01$). These results indicate that *Cryptosporidium* infection might be a significant cause of diarrhea in patients with HBV-associated ACLF was coincided with Yu *et al.* in a study done in China [34].

In our study, all positively stained fecal sample slides from ACLF patients, detected by MZN, contained only a few oocysts (1-5 oocysts/field of magnification) and none of 21 ACLF patients with *Cryptosporidium* infection suffered intractable diarrhea or watery diarrhea. These results indicate that the clinical significance of *Cryptosporidium* infection in these patients needs to be further investigated. ACLF patients with *Cryptosporidium* infection

received no specific anti-*Cryptosporidium* treatment; it is necessary to evaluate whether early treatment of *Cryptosporidium* infection can shorten the hospitalization time and promote improvements in liver function.

6 Conclusion

The prevalence of *Cryptosporidium* infection in patients with HBV-associated ACLF was markedly high compared with the prevalence in CHB patients and in children with diarrhea. *Cryptosporidium* infection does not appear to be problematic in patients with HVB-associated ACLF Unlike the negative effect *Cryptosporidium* infection has on AIDS patients; However, *Cryptosporidium* infection might be a significant cause of diarrhea in patients with HBV-associated ACLF. The clinical significance of this parasitic infection needs to be further studied.

6.1 Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

6.2 Acknowledgements

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