Original Article

Is cryptosporidiosis a leading cause of environmental enteric dysfunction (EED)? An *in vivo* study in experimentally infected mice

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ABSTRACT

Background: Environmental enteric dysfunction (EED) is a serious medical condition associated with malnutrition. It is an important cause of pediatric morbidity and mortality worldwide. Meanwhile, cryptosporidiosis is a major cause of malabsorption leading to enteropathy and stunted growth.
Objective: To investigate whether cryptosporidiosis causes EED. A secondary objective is to explore the validity of biomarkers recommended for the diagnosis of EED on a histopathological basis.
Material and Methods: Forty male Swiss albino mice were divided into four groups (10 mice each): GI (IC): immunocompetent uninfected; GII (ICI): immunocompetent infected; GIII (IS): immunosuppressed uninfected; GIV (ISI): immunosuppressed infected. Mice of each group were euthanized at two-time intervals, 7- and 14 days post infection (dpi). Stool samples were microscopically examined for oocyst counts and assessment of fecal EED markers including fecal myeloperoxidase (fMPO) and fecal alpha-1-antitrypsin (fA1AT). Serum samples were analyzed for intestinal fatty acid binding protein (I-FABP). Histopathological correlation was performed using microscopic examination of stained ileal sections.
Results: Cryptosporidiosis significantly increased fMPO, fA1AT and serum levels of I-FABP in the infected groups compared to the uninfected groups ($P \leq 0.05$). These parameters were significantly higher in ISI mice in comparison to ICI mice. The histopathological changes were like those previously reported in EED cases. In addition, histopathological scoring showed a significant positive correlation with various markers examined for EED at both 7- and 14 dpi ($P \leq 0.001$).
Conclusion: Cryptosporidiosis is a potential leading cause of EED. Further studies are recommended to confirm usefulness of these noninvasive markers as efficient predictors of EED rather than intestinal biopsy.
Keywords: Cryptosporidiosis; EED; fA1AT; fMPO; I-FABP.

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INTRODUCTION

The EED is a common subclinical intestinal illness in children residing in low- and middle-income nations[1]. In fact, EED is one of the leading causes of malnutrition linked to delayed oral vaccination response, poor linear growth, and compromised cognitive function[2]. The track from EED to malnutrition and associated problems is attributed to malabsorption and chronic inflammation resulting from pathogens associated with intestinal barrier dysfunction[3]. Notably, parasitic intestinal infections represent a serious threat to public health, especially in developing nations[4]. In addition, chronic intestinal infections cause imperfect changes in the structure and function of the intestines, hindering a child's growth[5].

In children under two, cryptosporidiosis is the second, most prevalent cause of moderate-to-severe diarrhea, after Rotavirus. It is also a major global cause of mortality, especially in immunocompromised hosts and during waterborne outbreaks[6]. In Egypt, 10.0–16.7% of children with diarrhea had cryptosporidiosis[7]. *Cryptosporidium* spp. are recognized as an opportunistic disease-causing, protozoa-associated diarrhea, and intestinal problems in both immunocompromised patients and immunocompetent individuals[8]. These protozoans can directly compromise the intestinal barrier by attaching to cell surface molecules, or by disrupting tight junctions as well as cell cytoskeletons, causing cell damage and death. Cryptosporidiosis is associated with increased gut inflammation and loss of villus architecture resulting in enteropathy and stunted growth[4].

Nowadays, the most reliable method for diagnosing EED is intestinal biopsy; yet it is an invasive procedure. Consequently, biomarker panels were proposed to evaluate EED[5]. These biomarkers include: 1) intestinal absorption, e.g., α-1-antitrypsin (A1AT) in stool or duodenal aspirates, reflecting protein loss and increased permeability; 2) enterocyte mass turnover,

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or injury, e.g., intestinal fatty acid binding protein (I-FABP), an epithelial protein located at villus tips where its high levels indicate recent intestinal injury; 3) mucosal inflammation, e.g., fecal myeloperoxidase (fMPO)[9]. However, it is necessary to confirm EED prediction by identifying histopathological changes in biopsy specimens[10]. Unfortunately, most relevant research is of small sample size and without histological correlation.

Therefore, the objectives of the current study were to determine whether cryptosporidiosis causes EED and to explore the validity of biomarkers recommended for EED prediction on a histopathological basis.

MATERIAL AND METHODS

This experimental descriptive analytical study was conducted at Theodor Bilharz Research Institute (TBRI) during the period from January to March, 2024.

Study design: The relationship between EED markers and cryptosporidiosis was examined in an experimental animal model. Over the course of the experiment, mice were weighed, and fecal pellets were collected. Mice from each of the four study groups were euthanized at two-time intervals, 7th and 14th dpi. Within each group, five mice were euthanized at each time interval, and stool samples were collected for both oocyst count and measurement of EED fecal markers. Serum samples were collected for assessing I-FABP. Additionally, ileal sections of each mouse were prepared for histopathological evaluation.

Sample size calculation: The sample size was determined based on the study objectives and design. According to Arifin and Zahiruddin[11], we used resource equation approach (n= 20/K+1) to estimate the maximum number of mice in each group where n = number of mice per group, and k = number of groups. The sample size of 6 mice was increased to 10 mice to avoid 20% dropout rate.

Experimental animals: Forty male Swiss albino mice, aged 6-8 w weighing 20-23 g, and raised in the laboratory were supplied from the Biology Supply Center of Theodor Bilharz Research Institute (TBRI) in Giza, Egypt. Prior to the experiment, mice were investigated for three successive days to ensure that they were pathogen free[12]. The animals were housed in TBRI animal house, and kept at a consistent room temperature of 22±2°C. The customary food and water were freely available to the animals.

Study groups: The study utilized four equal groups of mice, in which the first two immunocompetent groups (GI, and GII) included non-infected (IC), and infected (ICI) mice. Groups III (IS, uninfected) and IV (ISI, infected) were immunosuppressed chemically using synthetic dexamethasone (Dexazone 0.5 mg, purchased from Kahira Pharmaceuticals and Chemical Industries Company, Cairo, Egypt). It was administered orally using an esophageal tube at a dose of 0.25 µg/g/day successively for 14 d prior to infection and continued daily throughout the experiment[12].

Mice infection: *Cryptosporidium* oocysts were obtained from TBRI. Freshly prepared un-excysted *Cryptosporidium* oocysts in a recently vortexed solution (10^5 oocysts per mouse) in 100 µl PBS were orally fed by gavage into the stomach of each infected mouse. Oral gavage of an equivalent µl of PBS was administered concurrently to control animals. A 12-h fasting was necessary prior to inoculation to facilitate the infection process[13].

Assessment of *Cryptosporidium* oocyst shedding: Up until the end of the experiment, fresh fecal pellets were individually collected beginning on the first day and every three days. After being homogenized and suspended in 10% formalin, samples were stained with MZN. Oocysts were counted using a hemocytometer[14].

Assessment of EED markers[1]: The concentration of fMPO and fA1At were determined using ELISA commercially available EMMPO (Thermo Fisher Scientific Inc, USA), and commercially available ab205088 (Abcam, China) kits, respectively. Additionally, blood samples (~2 ml) were withdrawn by cardiac puncture. Sera were retrieved, frozen, and kept in -20°C until used to determine I-FABP level using mouse FABP2/I-FABP ELISA kit (NBP2-82214) obtained from R&D system, Minneapolis, MN, USA.

Animals' euthanasia: On 7- and 14 dpi, mice were euthanized through cervical dislocation after isoflurane[15]. To remove food particles, ice-cold saline was pumped through the intestinal lumens of euthanized mice.

Histopathological correlation: One cm of the terminal ileum was excised from each mouse, fixed in 10% neutral buffered formalin, dehydrated in ascending grades of ethanol, followed by immersion in xylene, and embedding in paraffin. Paraffinized blocks were cut into 4 µ thin sections, mounted on clean glass microscopic slides, and stained by H&E stain for microscopic examination[16]. The histopathological scoring was done according to Ledwaba *et al*.[17]. The level of cell infiltration and epithelial tissue damage was quantified from 0 to 3, where 0: no damage, 1: mild, 2: moderate, and 3: extensive. The sum of the two assessed factors was used to calculate the overall histological damage score. The slides were examined using a multi-head microscope (Olympus SC100).

Statistical analysis: Data were collected, tabulated, statistically analyzed using an IBM personal computer with Statistical Package of Social Science (SPSS) version

20. Armonk, NY and Epi Info 2000 programs were used. Quantitative data were presented in the form of mean±SD, range, median and interquartile range, and qualitative data were presented in the form of numbers and percentages (%). Student's *t*-test was used for comparison between two groups having quantitative parametric variables. For comparing the same group at different times (7- and 14-dpi), paired *T* test was used for parameter quantitative variables. One-way ANOVA test (*F*) was used for comparison between more than two groups having quantitative parametric variables. Pearson correlation coefficient was used to study the correlation between parametric quantitative variables. Statistical significance was considered when $P \leq 0.05$.

Ethical consideration: International ethical principles were followed in all aspects of animal care and procedures. The study protocol, IRB No. 2/2024PARA5, was approved by the Menoufia University Faculty of Medicine's Ethics Committee for Scientific Research.

RESULTS

Weight changes: Cryptosporidiosis decreased weight in immunocompetent and immunosuppressed mice. The weight loss percentage in the ICI group was significantly increased compared to the normal control group (IC) at both 7- and 14 dpi ($P \leq 0.001$). The ISI mice also showed significant weight loss compared with IS mice at both time intervals ($P \leq 0.001$) (Fig. 1).

Fecal MPO: Cryptosporidiosis significantly increased fMPO in the infected groups compared to the uninfected groups. At 7 dpi, there was a statistically significant difference between IC and ICI groups ($P \leq 0.001$) and between IS and ISI groups ($P \leq 0.001$). It was observed that the increase in ISI mice was statistically higher than ICI mice ($P \leq 0.001$). At 14 dpi, there was a statistically significant difference between IC and ICI groups ($P \leq 0.001$) and between IS and ISI groups ($P \leq 0.001$). It was observed that the increase in ISI mice was statistically higher than ICI mice ($P \leq 0.001$). The mean fMPO level in ICI group at 14 dpi (6.3±0.99) was statistically higher than at 7 dpi (3.5±0.92) ($P \leq 0.001$) while in ISI group, no significant difference was recorded between 7- and 14 dpi (Table 1 and Fig. 3).

Fecal A1AT: This showed a significant increase in infected than uninfected groups. At 7 dpi, there was a statistically significant difference between IC and ICI groups ($P \leq 0.001$) and between IS and ISI groups ($P \leq 0.001$). It was observed that the increase in ISI mice was statistically higher than ICI mice ($P \leq 0.001$). At 14 dpi, there was a statistically significant difference between IC and ICI groups ($P \leq 0.001$) and between IS and ISI groups ($P \leq 0.001$), where the increase in ISI mice was statistically higher than ICI mice ($P \leq 0.001$). There was no significant difference in the mean fA1AT level in relation to time of euthanasia in all groups (Table 1 and Fig. 3).

Intestinal FABP: The mean serum levels of I-FABP were elevated in infected groups than in uninfected groups. In ICI mice, it was 2.0±0.29 and this was significantly higher than in IC ($P \leq 0.001$). In ISI, it was 3.9±0.35, also significantly higher than IS ($P \leq 0.001$). Comparing both infected groups, the level in ISI was significantly higher than ICI ($P \leq 0.001$). Significant difference of $P=0.01$ and $P=0.004$ was recorded in the mean serum levels of I-FABP in relation to time of euthanasia of infected ICI and ISI groups respectively (Table 1 and Fig. 3).

Histological injury score: Mice infected with *Cryptosporidium* exhibited changes in the intestinal structure in the form of severe epithelial layer damage, distortion of the villus architecture, loss of glandular integrity and presence of many oocysts within the crypts of the mucosa. There was also inflammatory cell infiltration in the ileum and increased intraepithelial lymphocytes, resulting in higher histopathologic scores compared to uninfected mice ($P<0.05$).

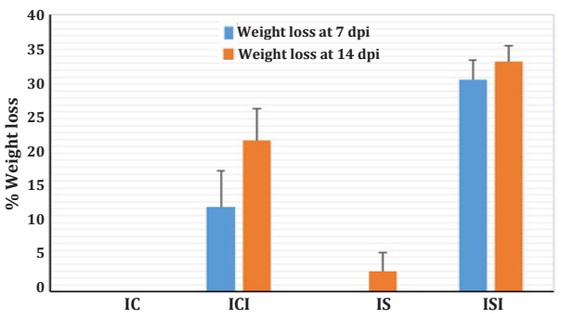

Fig. 1. Percentage of body weight loss in all studied groups.
IC: Immunocompetent uninfected;
ICI: Immunocompetent infected;
IS: Immunosuppressed uninfected;
ISI: Immunosuppressed infected.

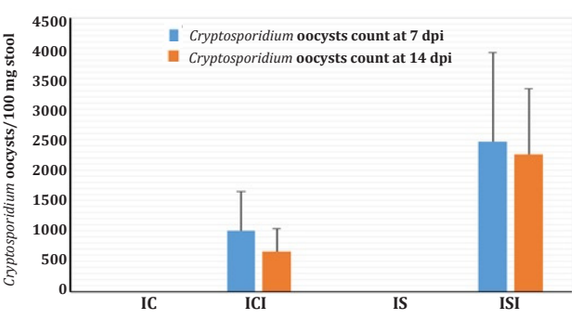

Fig. 2. *Cryptosporidium* oocysts count in all studied groups.
IC: Immunocompetent uninfected;
ICI: Immunocompetent infected;
IS: Immunosuppressed uninfected;
ISI: Immunosuppressed infected.

The ICI mice euthanized at 7 dpi showed mild degree of villous distortion with presence of many oocysts within the crypts of the mucosa. At 14 dpi, there was moderate distortion of villi, infiltrated by plasma cells and lymphocytes as well as presence of few oocysts in relation to the surface epithelium. The ISI mice showed shortness of villi with presence of many oocysts within the crypts of the mucosa at 7 dpi, while at 14 dpi they showed highly distorted villi extensively infiltrated by plasma cells and lymphocytes as well as presence of many oocysts in relation to the surface epithelium (Fig. 4).

The histopathological scores for ICI and ISI infected groups were higher compared to those in the normal control group, with a statistically significant difference (P<0.05). The histological score in the ICI group was lower compared with that in the ISI group (P<0.05) at both time intervals (Fig. 4).

Correlation between histopathological scoring and different EED markers: Histopathological scoring showed a significant positive correlation with various markers examined for EED at either 7- or 14 dpi (P≤0.001) (Fig. 5). As regards weight changes, percentage of weight loss showed a significant positive correlation with the histopathological score throughout the study [r= 0.894 (P≤0.001), r= 0.957 (P≤0.001); respectively]. Concerning oocyst shedding, a significant positive correlation with histopathological score at both times of euthanasia was recorded [r= 0.906 (P≤0.001), r= 0.786 (P≤0.001); respectively] (Fig. 6).

Table 1. Comparison of different EED markers between different groups in relation to time.

Study groups	Fecal MPO (µg/g stool) Mean±SD		Fecal A1AT (mg/g stool) Mean±SD		Serum I-FABP (pg/g stool) Mean±SD	
	7 dpi	14 dpi	7 dpi	14 dpi	7 dpi	14 dpi
Group I (IC)	0.18±0.053	0.21±0.082	0.19±0.088	0.20±0.90	0.74±0.048	0.76±0.05
Paired *t* test	0.575		0.376		0.408	
***P* value**	0.596		0.726		0.704	
Group II (ICI)	3.5±0.92	6.3±0.99	61.3±7.9	74.8±6.2	2.0±0.29	3.5±0.65
Paired *t* test	3.6		2.61		4.16	
***P* value**	0.022*		0.056		0.014*	
Group III (IS)	0.31±0.17	0.30±0.14	0.36±0.12	0.38±0.16	1.05±0.22	1.01±0.16
Paired *t* test	0.109		0.389		0.228	
***P* value**	0.918		0.717		0.831	
Group IV (ISI)	7.9±1.2	12.4±3.1	85.6±8.5	108.4±19.8	3.9±0.35	5.2±0.42
Paired *t* test	2.72		2.09		5.81	
***P* value**	0.053		0.104		0.004*	

*: Significant (P≤0.05).

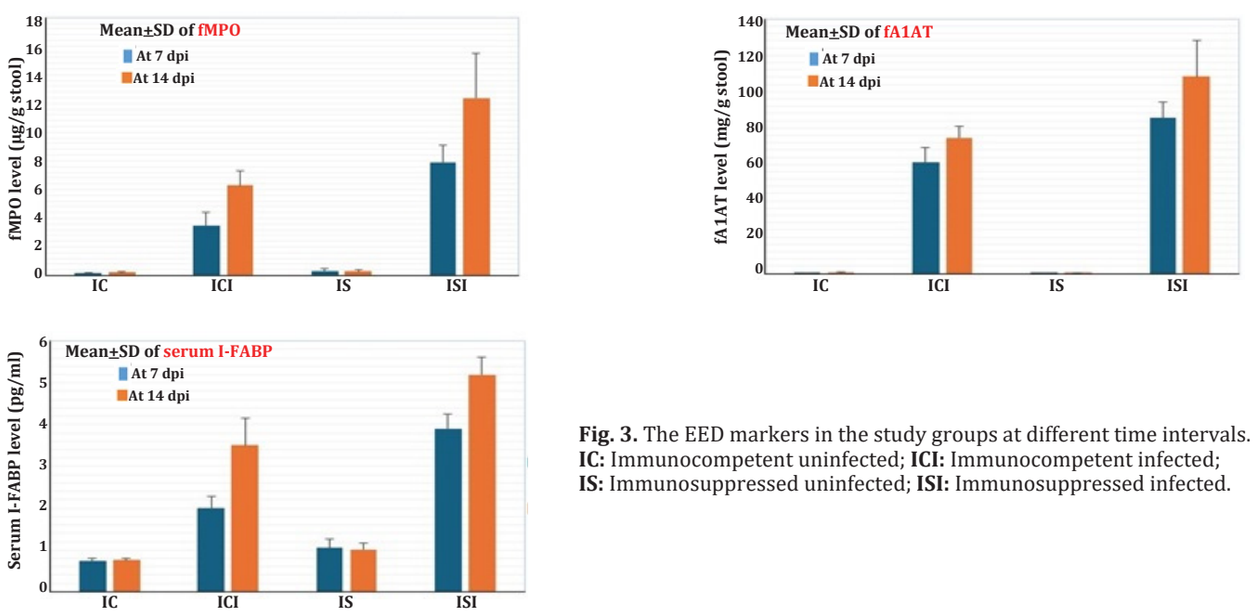

Fig. 3. The EED markers in the study groups at different time intervals. **IC:** Immunocompetent uninfected; **ICI:** Immunocompetent infected; **IS:** Immunosuppressed uninfected; **ISI:** Immunosuppressed infected.

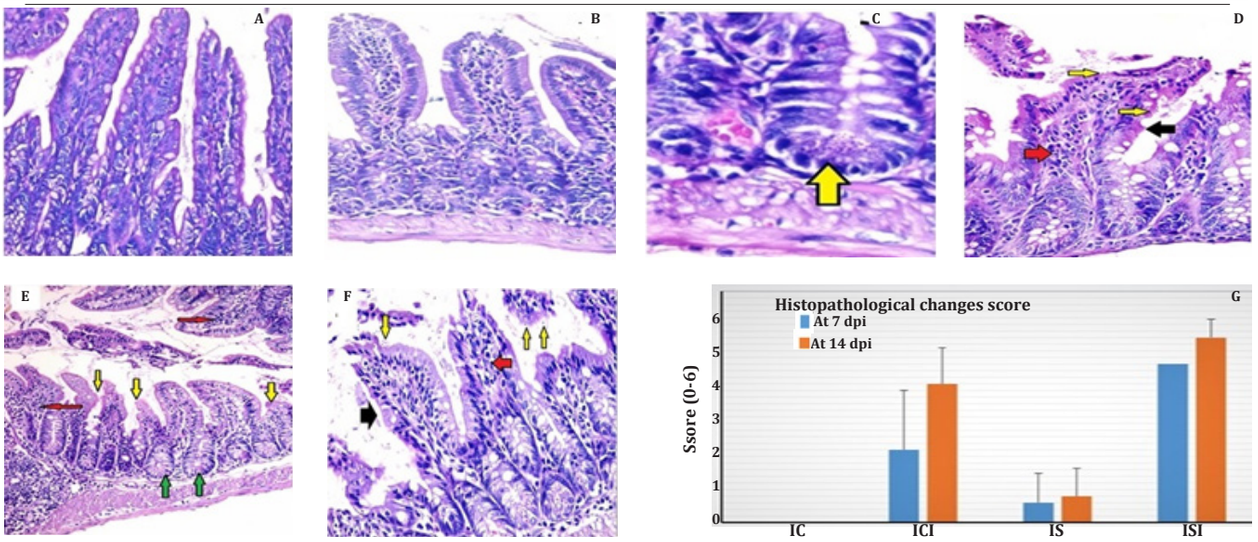

Fig. 4. Histopathological sections of ileum in the study groups. **(A)** normal histology of control negative mice, the inset showed normal villous architecture; **(B, C)** infected immunocompetent mice euthanized at 7 dpi showing mild degree of disturbed villous architecture with blunted tips and shortness of villi **(B)**, and presence of many oocysts within the crypts (yellow arrow) of the mucosa **(C)**; **(D)** infected immunosuppressed mice euthanized at 7 dpi showing moderate degree of disturbed villous architecture with blunted tips and shortness of villi (black arrow) infiltrated by plasma cells and lymphocytes (red arrow) as well as presence of many oocysts in relation to the surface epithelium (yellow arrows); **(E)** infected immunocompetent mice euthanized at 14 dpi showing moderate degree of distorted villous architecture with blunted tips and shortness of villi, ulceration and focal sloughing of villous tips (yellow arrows), infiltration by plasma cells and lymphocytes (red arrows) with many oocysts within the crypts of the mucosa (green arrows); **(F)** infected immunosuppressed mice euthanized at 14 dpi showing moderate to extensive degree of distorted villous architecture with blunted tips and shortness of villi (black arrow), ulceration and focal sloughing of villous tips together with heavy inflammatory cells infiltration of plasma cells and lymphocytes (red arrow) as well as presence of many oocysts in relation to the surface epithelium (yellow arrows). (Magnification: 200× for all except for (C) 400x); **(G)** histopathological damage score. Data are expressed as mean±SD with statistically significant difference (P<0.05).

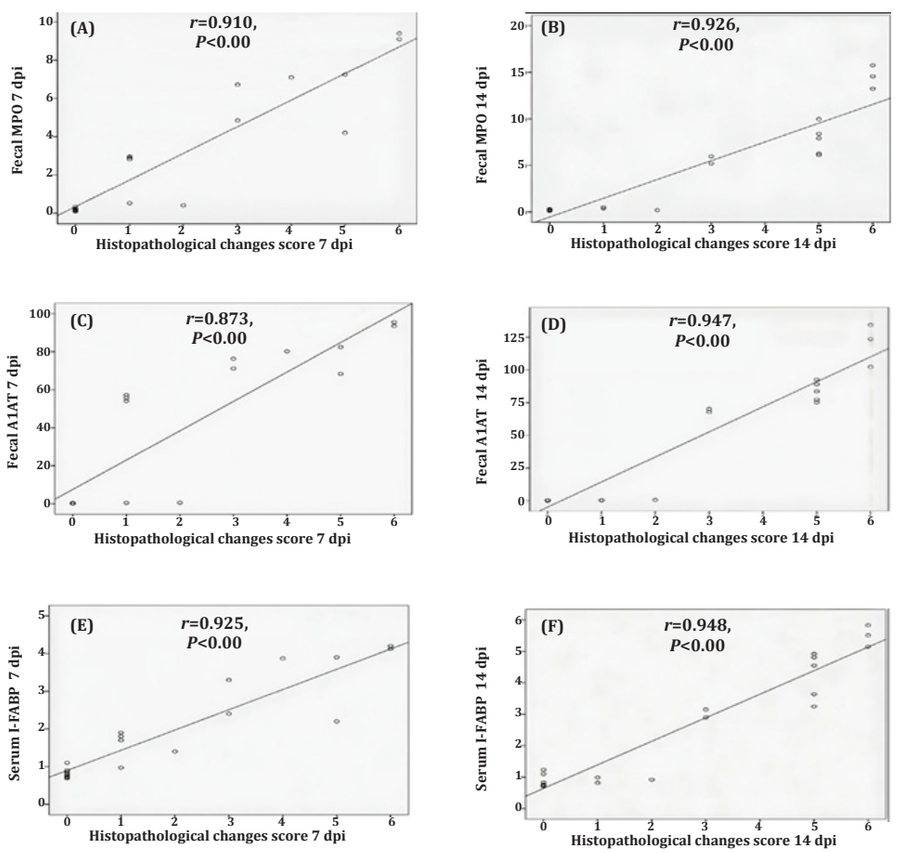

Fig. 5. Correlation between histopathological scoring and fMPO at 7 dpi **(A)**; fMPO at 14 dpi **(B)**; fA1AT at 7 dpi **(C)**; fA1AT at 14 dpi **(D)**; serum I-FABP at 7 dpi **(E)**; serum I-FABP at 14 dpi **(F)**.

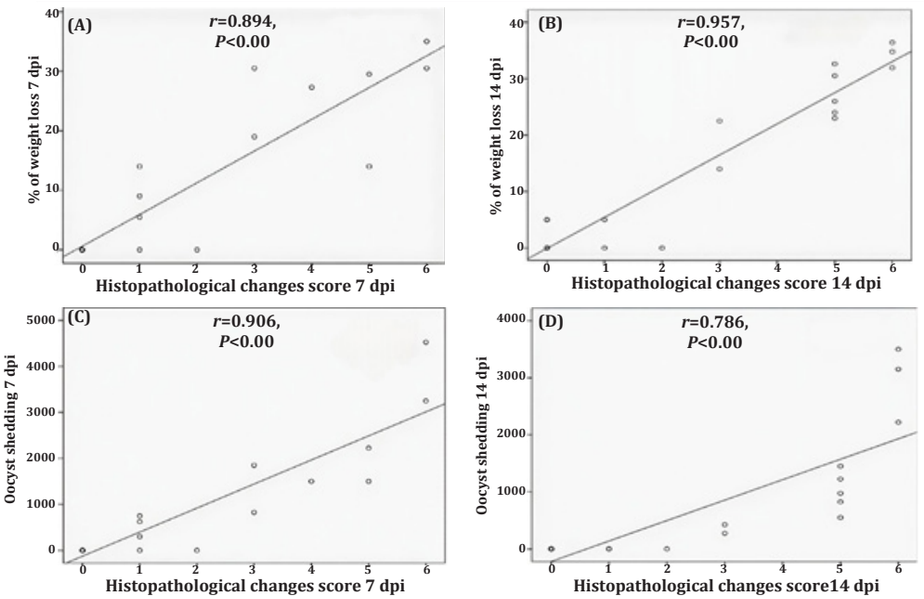

Fig. 6. Correlation between histopathological scoring and percentage of weight loss at 7 dpi **(A)**; percentage of weight loss at 14 dpi **(B)**; oocysts shedding at 7 dpi **(C)**; oocysts shedding at 14 dpi **(D)**.

DISCUSSION

In fact, EED is a persistent, subclinical disease that often has no gastrointestinal symptoms but results in a chronic, persistent inflammatory state and ineffective absorption of nutrients. Cryptosporidiosis is associated with increased gut inflammation and loss of villus architecture[18] allowing the spread of microorganisms into the circulation which triggers the release of acute-phase reactants, and inhibits insulin-like growth factor-1, which suppresses linear growth[19]. The present study showed that cryptosporidiosis could be a major cause of EED. It affected the weight of mice with histopathological changes such as those recorded in cases of EED. Besides, EED markers, both in stool and serum, were significantly elevated with a positive correlation to histopathology findings. Furthermore, the immune status of the mice affected the occurrence and severity of the condition. Cryptosporidiosis clearly resulted in reduction of mice weight in both immunocompetent and immunosuppressed groups. These findings were in accordance with previous reports[20,21]. Contrarily, Oriá *et al*.[13] reported that protein undernutrition and cryptosporidiosis led to impaired growth and weight gain.

Admittedly, the inflammatory response is influenced by the invasiveness of the parasite and the host's immune status, which in turn impacts the parasite load and the span of infection[22]. In the present study, the oocyst shedding was greater in immunosuppressed mice. Likewise, the protective role of the immune system in controlling cryptosporidiosis has been reported in previous studies[14,23]. However, Bolick *et al*.[21] observed that peak oocysts shedding occurred approximately 5 dpi, coinciding with up to 20% weight loss in infected mice with protein deficiency.

Since there are no obvious acute symptoms with EED, intestinal biopsy, which is invasive, expensive, and requires technical expertise, has historically been the gold standard for diagnosing abnormalities in intestinal tissue[18]. Most research and clinical settings do not allow such an invasive diagnosis, and it is also limited by the possibility of inadequate sampling because the sample taken from the biopsy cannot represent the entire intestine. In the absence of these interventional methods, diagnosing EED proved difficult[24]. As a result, studies question the use of commonly available, less invasive biomarkers of the underlying diseases of EED to aid in diagnosis[18]. Several biomarkers have been used in the diagnosis and prognosis of EED. Our study is concerned with fMPO as an indicator of mucosal inflammation, fA1AT as an indicator of intestinal permeability, and protein loss and serum I-FABP as an indicator of recent intestinal injury.

Myeloperoxidase is a peroxidase contained within lysosomal azurophilic granules in neutrophils and is a marker of neutrophil degranulation, which can be detected in stool[25,26]. Because fMPO is readily available and has the potential for use in a murine enteropathy model, we used it as a primary fecal marker test for intestinal inflammation[27]. In addition, fMPO is stable in feces as it is not susceptible to oxidative proteolysis and supernatants can contain adequate levels for up to 3-7 d at 4°C[28,29]. In the present investigation, cryptosporidiosis resulted in elevated fMPO levels that were higher in immunosuppressed mice compared with immunocompetent mice. This is consistent with

Bolick *et al*.[21] who reported that cryptosporidiosis with protein deficiency significantly increased fMPO than in uninfected groups. Similarly, a prior study demonstrated a correlation between increased fMPO and entero-pathogens in non-diarrheal stool samples[25]. Moreover, another study claimed that children from low- and middle-income countries have higher than average levels of fMPO[27]. Contrary to our results, Fahim *et al*.[1] found that the presence of *Cryptosporidium* oocysts in non-diarrheal stool samples was not connected to any fecal indicators or EED score. Besides, our elevated levels of fMPO were associated with increased histopathological score. In accordance with our results, Peterson *et al*.[30] reported that elevated levels of fMPO correlated with disease activity in ulcerative colitis (UC) patients.

When a *Cryptosporidium* oocyst binds to epithelial cell surface molecules, it results in damage and apoptosis of the cells, or it can break down tight junctions and the cytoskeleton of cells, which may directly disrupt the intestinal barrier[4]. The A1AT is a 52-kDa glycoprotein serine protease inhibitor that is produced predominantly by hepatocytes and found in the bloodstream at constant levels[31]. However, it is expelled from the intestine during protein-losing enteropathy so it can be used as an indicator of intestinal permeability[32]. Furthermore, it is a relatively inexpensive test[26]. The present work confirmed that fA1AT levels were elevated in *Cryptosporidium*-infected mice and significantly correlated with the histopathological score. The ongoing findings are in agreement with a study that observed significant association of fA1AT in giardiasis affecting children <2 y[1]. Similarly, the presence of entero-pathogens in non-diarrheal stool samples was linked to higher fA1AT[25].

It is well known that tissues involved in the uptake and utilization of fatty acids contain low molecular weight cytosolic proteins called fatty acid binding proteins (FABPs). It has been suggested that the presence of FABPs in the bloodstream or urine is a sign of early enterocyte or mucosal injury[33]. Due to their early elevation after intestinal cell injury and specificity to intestinal diseases, these FABPs are valuable as biomarkers of the gastrointestinal tract[34]. In our study, cryptosporidiosis resulted in an elevation in I-FABPs associated with a significant elevation of histopathological score. Consistently, Rajamanickam *et al*.[35] reported elevated levels of I-FABP in subjects infected with hookworm and *S. stercoralis*. On the other hand, Hasan *et al*.[36] declared that I-FABP is negatively associated with linear growth of Bangladeshi children.

Regarding the histopathological results, the microscopic findings in infected groups in the present study were like those previously reported by Watanabe and Petri[10] in cases of EED including distorted blunted villi, deeper crypts, fewer compartmental characteristics, increased epithelial cell shedding, and increased cellular infiltrates that are primarily composed of plasma cells and lymphocytes. Our study results were consistent with those findings. The increased intensity of infection associated immune suppression was reflected in the pathology and the calculated histopathological scores. The histopathological scores were parallel to the biochemical findings of this study. Our valuable remark on this work is the correlation between the histopathological findings and the non-invasive biomarkers of EED in cryptosporidiosis. Here, we found that the severity of pathological changes correlated positively with all tested EED markers. In addition, the severity of pathological changes was positively correlated with the severity of infection represented by oocyst shedding and the percentage of weight loss throughout the study period.

In conclusion, our study recorded that cryptosporidiosis is significantly associated with elevated fMPO and fA1AT concentrations in stools, and the earliest and highest response was recorded for serum I-FABP, and these markers are well correlated with the pathological severity. So, our study suggests that cryptosporidiosis could be a leading cause of EED and that these noninvasive markers could be a good predictor of EED rather than intestinal biopsy with further studies recommended to ascertain this.

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