CLINICAL AND EPIDEMIOLOGICAL STUDY

Cryptosporidium gastroenteritis in Egyptian children with acute lymphoblastic leukemia: magnitude of the problem

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Abstract

Purpose   *Cryptosporidium* species is considered to be an important cause of significant morbidity in immunocompromised individuals. A prospective case–control study of sporadic diarrhea due to *Cryptosporidium* infection was conducted on children with acute lymphoblastic leukemia (ALL).

Methods   Forty children with ALL on maintenance chemotherapy according to the Berlin–Frankfurt–Munster (BFM-90) protocol and 45 sex- and age-matched controls were studied. The ALL group included 25 patients with acute diarrhea and 15 without diarrhea, and the control group included 30 children with acute diarrhea and 15 without. Collected stool specimens were examined using modified Ziehl–Neelsen (MZN) and modified trichrome stains. Serum *Cryptosporidium Parvum* immunoglobulin G (IgG) antibodies were detected by enzyme-linked immunosorbent assay.

Results   *Cryptosporidium* oocysts, pathogenic Gram-negative organisms, *Giardia lamblia*, and *Entamoeba histolytica* were identified in the stool samples (fecal specimens) of six (24%), eight (32%), four (16%), and two (8%), respectively, of the 25 patients with ALL and actute diarrhea and in one (3%), two (6.5%), six (20%), and five (16.5%), respectively, of the 30 control patients with diarrhea. Serum IgG antibodies were positive in four of the six ALL patients and in one of the control group patients with *Cryptosporidium* diarrhea who tested positive for oocysts in the stool. Diarrhea duration and severity were greater in ALL patients with stool-positive *Cryptosporidium* oocysts than in those with non-*Cryptosporidium*-positive diarrhea ($p < 0.000$).

Conclusions   *Cryptosporidium* infection should be considered in children with ALL presenting with prolonged or severe watery diarrhea during chemotherapy, especially those treated with methotrexate and 6-mercaptopurine. Since *Cryptosporidium* is not routinely tested for in stool examination, a MZN stain is recommended.

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Introduction

During the past two decades, there has been a progressive improvement in the outcomes for children with acute lymphoblastic leukemia (ALL) in clinical trials, which has resulted in optimized post-induction treatment intensification

[1]. Due to the neurotoxicities of cranial irradiation, most ALL protocols are limited to the combination of intrathecal and intravenous methotrexate (MTX) as gold standard for central nervous system (CNS) prophylaxis. High doses of MTX have adverse effects, primarily neutropenia, mucositis, and myelosuppression [2]. The aim of current risk-directed therapy protocols is to improve not only ALL cure rates but also the patients' quality of life in terms of decreased acute morbidity and long-term sequelae [3]. Treatment-related toxicities represent a major health risk to children with ALL. Bacterial and viral infections, secondary to severe neutropenia and/or immunosuppression, contribute to significant morbidity, hospitalizations, and a 1.7–4.7% incidence of death in remission [4].

Gastroenteritis is common in cancer patients [5]. The diagnosis and treatment of moderate-to-severe diarrhea in patients with cancer is often a challenge because of the variety of infectious and non-infectious causes [6]. Patients with acute leukemia are susceptible to infection at an increased incidence. Neutropenic enterocolitis is an acute, life-threatening inflammation of the small and large bowel and is often seen in children with malignancies during periods of prolonged or severe neutropenia [7]. Patients on immunosuppressive therapy have an increased probability of acquiring parasitic infections, generally with a high degree of severity [8].

Cryptosporidium spp. are a leading cause of diarrhea, particularly persistent diarrhea in children in developing countries [9]. Parasitic infections that cause self-limited diarrhea in immune-competent patients may cause profuse diarrhea in immune-compromised individuals. In such patients, parasites may disseminate to other organs, such as the bronchi, liver, and bile ducts, producing symptomatology specific to the affected organ [10].

Cryptosporidium oocysts are present in 65–97% of surface water (i.e., rivers, lakes, and streams) tested throughout the USA. Transmission of the parasite occurs by the fecal–oral route through the ingestion of oocysts that are shed with the feces of infected hosts [11]. This transmission route together with the resistance of oocysts to environmental conditions and chlorine [12], a large parasite reservoir, and a low infective dose account for the risk of transmission by water [13].

The study reported here was designed to assess the magnitude of *Cryptosporidium* gastroenteritis in children with ALL who are in remission and on maintenance chemotherapy.

Patients and methods

This study was conducted in the Hematology/Oncology Clinic Children's Hospital, Ain Shams University. The Pediatrics Ethics Committee of the hospital approved the study and it was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. All care-givers of children provided their informed consent prior to the collection of 55 stool specimens from 25 children with ALL and diarrhea and 30 matched controls. Acute diarrhea was defined as three or more loose or watery stools within an 8-h period for <6 days prior to presentation. An episode was considered to have ended when the last day of diarrhea was followed by three consecutive non-diarrheal days. Dysentery was diagnosed when stools mixed with gross blood were observed or reported. Episodes that lasted for >14 days were defined as persistent. Thirty stool specimens were also collected from children without diarrhea: 15 were ALL patients in remission, and 15 were immuno-competent healthy children who were relatives or neighbors of the ALL children and matched for age, sex, social status, and residence. All enrolled children were from rural areas. At the time of specimen collection, families completed questionnaires for each subject in which they provided demographic details and clinical history. Vital signs (temperature, pulse, blood pressure) and the results of a physical examination (including signs of anemia, purpura, mouth ulcers, and signs of dehydration) were recorded. Patients with severe diarrhea and dehydration were treated in-hospital with rehydration measures and, when necessary, appropriate antibiotics were given, based on stool culture results. Children with *Cryptosporidium* diarrhea were treated for 10 days with azithromycin.

All children with ALL were in remission and on maintenance chemotherapy. None of the enrolled children had neutropenia based on complete blood counts. The Berlin–Frankfurt–Munster (BFM 90) chemotherapy protocol was used to treat ALL. Maintenance therapy was initiated 2 weeks after the end of re-induction. The scheduled dose of 6-mercaptopurine was 50 mg/m^2 a day given orally. MTX was given orally at a dose of 20 mg/m^2 once a week, with adjustments in dosage made in accordance with the white blood cell (WBC) count (target range $2–3 \times 10^9$/L). For all patients, the total duration of therapy was 24 months [14].

Fresh fecal specimens (3 samples from each child, on 3 consecutive days) were collected in clean, labeled, dry disposable plastic cups. All specimens were processed within 2 h of collection for bacterial and parasitological studies. Bacterial isolates were stored for subsequent identification. A direct smear was made for the detection of parasitic infestations [15]; the modified Ziehl–Neelsen (MZN) and modified trichrome stains were used to detect *Cryptosporidium* spp. [16] and microsporidia [17], respectively. In the active infection, *Cryptosporidium* oocysts appear as pink entities on a green background

following acid-fast staining; during a resolving infection, an increased number of "ghosts" (i.e., non-acid-fast oocysts) are observed (variable staining).

Enteropathogens were investigated by stool cultures. Fecal specimens were inoculated on blood agar, *Salmonella–Shigella* agar, MacConkey agar (Biofilchem, Roseto, Italy), sorbitol MacConkey agar, and *Yersinia* selective agar (Oxoid, Basingstoke, UK). Selective and differential media were used for isolating *Salmonella*, *Shigella*, *Yersinia spp.*, and *Escherichia coli.* All plates were examined, and putative colonies of enteropathogenic bacteria were identified by standard biochemical methods (API 20E system; bioMérieux, Marcy-l'Étoile, France) [18]. Stool samples were not examined for viruses.

Serum immunoglobulin G (IgG) antibodies against *Cryptosporidium parvum* oocysts were detected using an enzyme-linked immunosorbent assay (ELISA) (A8667; Sigma-Aldrich, St. Louis, MO). The cut-off value for the ELISA was determined by estimating the mean optical density of the negative control samples $+$ 3 standard deviations (SD).

Statistical analysis

The Statistical Package for the Social Sciences ver. 9 (SPSS, Chicago, IL) was used for statistical analysis. Differences between groups were tested by analysis of variance (ANOVA), chi-square test (χ^2), and the Fisher's exact two-tailed test was used to detect whether there was a significant association between the different categorical variables. The p value used to indicate the level of significance was set at $p < 0.05$.

Results

The mean age of the children enrolled in the study was 8.25 $\pm$ 3.5 (range 2–15) years. The mean age of the ALL children with *Cryptosporidium* diarrhea ($n = 6$) was 8.4 $\pm$ 0.66 years, which was comparable to the mean age of 6.9 $+$ 1.79 years of the ALL children with non-*Cryptosporidium* diarrhea ($n = 19$) ($F = 4.04$, $p = 0.056$). None of the studied ALL children had either neutropenia or eosinophilia at the time of diarrhea.

Cryptosporidium oocysts were detected more often in stool specimens of ALL children with diarrhea than in those of the control group with diarrhea [6/25 (24%) vs. 1/30 (3%), respectively; $\chi^2 = 5.24$, $p = 0.039$). Pathogenic Gram-negative organisms were also detected more often in the stool specimens of children with ALL and diarrhea (8/25, 32%; 2 *Salmonella typhi*, 1 *Campylobacter jejuni*, 2 *Shigella* spp., 2 enterotoxigenic *Escherichia coli* (ETEC), 1 *Yersinia enterocolitica*] than in the control

group with diarrrhea (2/30, 6.5%; 2 with ETEC; $\chi^2 = 5.9$, $p = 0.032$). The prevalence of *Giardia lamblia duodenalis/intestinalis* (*G. lamblia*) did not differ between children with ALL and diarrhea (4/25, 16%) and the control group with diarrhea (6/30, 20%) ($\chi^2 = 0.15$, $p = 0.74$). The *Entamoeba histolytica/dispar* complex was found in 2/25 (8%) children with ALL and diarrhea and in 5/30 (16.5%) control group children with diarrhea. *Cyclospora* and microsporidia were not detected in the stool specimens. Serum IgG was positive in 4/6 ALL children whose stool specimen tested positive for *Cryptosporidium* and in the one control child with *Cryptosporidium* diarrhea.

No organism were detected in the stool specimens of 5/25 ALL children with diarrhea, 16/30 control children with diarrhea, and in all children without diarrhea whether with ALL or not (healthy control) (Fig. 1).

The two ALL children whose serum tested negative for *Cryptosporidium* antibodies but whose stool tested positive for *Cryptosporidium* oocysts had recurrent attacks of neutropenia, necessitating interruption of the chemotherapy prior the attack of diarrhea. One of these patients had three severe attacks of mouth ulcers and abdominal pains due to MTX therapy (resolved with leucovorin).

When ALL children with diarrhea were compared to the control group with diarrhea, the prevalence of fever was significantly higher in the ALL children with diarrhea (36 vs. 26%, $p < 0.001$) and the duration of acute diarrhea was significantly longer (20.16 $\pm$ 6.3 days vs. 10.8 $\pm$ 5.8 days;
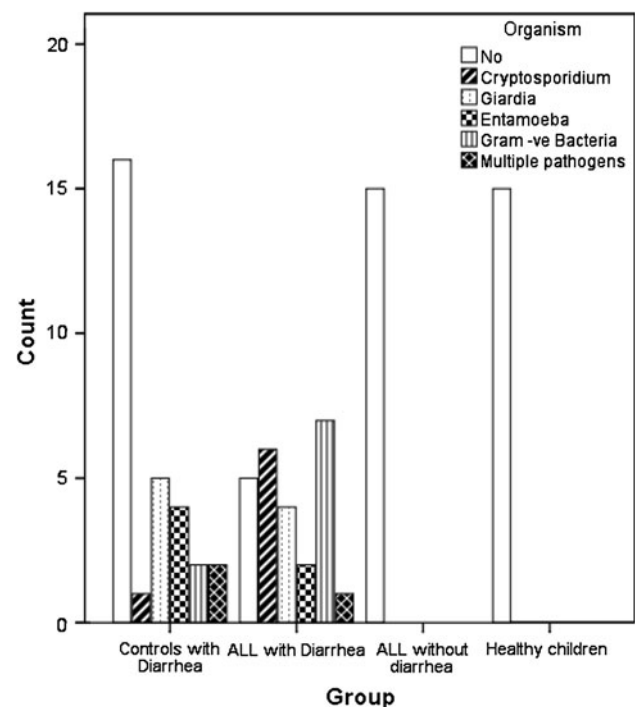
Fig. 1 Organisms detected in stools from children with diarrhea and control groups. *ALL* Acute lymphoblastic leukemia

$p < 0.001$). Abdominal colic, vomiting, frequency of bowel motions, watery diarrhea, and dehydration were notably higher in ALL children with diarrhea than in the control group with diarrhea. There was no difference in the frequency of fever, vomiting, colic, or stool consistency between ALL children with *Cryptosporidium* and non-*Cryptosporidium* diarrhea. ALL children with *Cryptosporidium* diarrhea had frequent daily diarrheal motions, severe dehydration, and a longer duration of diarrhea compared to children with non-*Cryptosporidium* diarrhea (Table 1).

Twenty-one ALL children with diarrhea were hospitalized, of whom five had *Cryptosporidium* diarrhea [1 with persistent diarrhea (>14 days), 2 with persistent high fever, 2 with severe emesis]. All five children with *Cryptosporidium* diarrhea were treated with nitazoxanide. The two sero-negative children for *Cryptosporidium* were difficult to treat and responded to azithromycin with stool clearance. Seven ALL children with Gram-negative pathogenic bacteria were hospitalized because of fever, colic, deficient oral intake, and one case of suspected acute appendicitis. Two ALL children with *Entamoeba histolytica*, three with *Giardia lamblia*, three with no pathogen detected in their stools, and one with suspected acute appendicitis were hospitalized. All hospitalized children received intravenous fluid therapy and antimicrobial therapy according to the stool culture and sensitivity. Among three of the five ALL children with no detected bacterial pathogen, two improved with ganciclovir treatment. There were two cases of suspected appendicitis among ALL children. One such patient had MTX-induced typhlitis with increased bowel wall thickness based on ultrasonography findings. This was successfully treated with bowel rest (withholding of oral food and fluids) and vancomycin. The second patient had *Yersinia enterocolitica* enteritis and improved with ceftriaxone treatment. Six ALL children without diarrhea were hospitalized because of fever. The control cases with diarrhea were managed in the rehydration unit as outpatients, except for three who needed hospitalization: one with persistent diarrhea and two with severe dehydration and vomiting. The child with *Cryptosporidium* diarrhea in the control group did not require hospitalization as the diarrhea resolved spontaneously without antimicrobial treatment after 3 days, as evidenced by clinical improvement and stool clearance.

Discussion

Cryptosporidium oocysts were detected in 6/25 (24%) diarrheal stool specimens from children with ALL. In a previous study, there was a 17.5% incidence of *C. parvum* oocysts in immune-compromised children with diarrhea, 18.3% incidence in children suffering from protein energy malnutrition, and 7.3% incidence in immune-competent children [19]. Under-notification of infections caused by *Cryptosporidium*

Table 1 Comparison of clinical symptoms between ALL children with *Cryptosporidium* and non-*Cryptosporidium* diarrhea

Clinical data	ALL patients with *Cryptosporidium* diarrhea ($n = 6$)	ALL patients with non-*Cryptosporidium* diarrhea ($n = 19$)	p (χ^2 test)
Colic			
Yes	1	4	0.054
No	5	15	0.81
Vomiting			
Yes	2	2	1.76
No	4	17	0.18
Fever			
Yes	2	7	0.024
No	4	12	0.87
Duration (mean $\pm$ SD)			
Days	20.33 $\pm$ 1.86	9.57 $\pm$ 6.09	17.68[a]
			<0.001*
Frequency (mean $\pm$ SD)			
Motions/day	14.3 $\pm$ 0.51	9.63 $\pm$ 2.06	29.81[a]
			<0.001*
Consistency			
Watery	6	16	1.07
Mucus and blood	0	3	0.29
Dehydration			
Yes	3	1	6.79
No	3	18	0.009*

Data on variables are given as the number (n) of patients unless otherwise stated

ALL Acute lymphoblastic leukemia, *SD* standard deviation

* Significant at $p < 0.05$. Fisher's-exact two-tailed test was used to determine associations among variables

[a] For statistical analysis, the analysis of variance test was used in these cases; otherwise significance was determined using the chi-square (χ^2) test

raises the question of whether routine testing should be carried out in all cases of gastroenteritis in children [20].

In our study, diarrhea was more severe in ALL children with *Cryptosporidium* diarrhea: compared to the controls *Cryptosporidium* infections in ALL patients were significantly more severe and longer (up to 4 weeks). Similar results have been reported in other studies [21].

Among our patients, stool examination using MZN stain detected detect oocysts in six *Cryptosporidium*-positive stools of children with diarrhea. However, serum *Cryptosporidium* IgG tested positive in only four of ALL children with *Cryptosporidium*-positive stools for oocysts and in the immune-competent control child with *Cryptosporidium* diarrhea. Other studies have shown the value of stool examination using MZN stain in diagnosing *Cryptosporidium* (13/35) in immune-deficient cases, with ELISA detecting only 11 cases [22]. Stool examination by MZN stain and PCR was 100% sensitive in both HIV sero-positive and sero-negative patients, with the specificity of antigen detection being 92.2 and 96.3% in human immunodeficiency virus (HIV) sero-positive and sero-negative patients, respectively. Staining techniques have been found to be less sensitive than antigen detection methods and PCR for the detection of *Cryptosporidium* [23].

In our study, ALL patients were on maintenance chemotherapy. Chemotherapy, especially with MTX and 6-mercaptopurine, has deleterious effects on the gastrointestinal tract. Gwavava et al. [24] noted marked morphological abnormalities in the villus enterocytes by light and electron microscopy examination, with MTX treatment causing striking distention of the lateral basal intercellular spaces, cell vacuolation, and patchy necrosis. These pathological changes were most distinct when the MTX treatment was given 24–72 h before biopsy and may account for the mal-absorption associated with MTX treatment [24]. Toxicity of high-dose methotrexate (HD-MTX) is usually limited to a moderate myelosuppression, reversible mucositis, and moderate hepatic or renal dysfunction. These effects can be substantially reduced via hydration, urinary alkalinization, and the monitoring of renal function and MTX serum concentrations [25]. 6-Mercaptopurine (6 MP) toxicity can also cause neutropenia, diarrhea, and vomiting [26]. Although none of the ALL children in our study had neutropenia during their diarrheal episodes, the two ALL children whose serum tested negative for *Cryptosporidium* antibodies had recurrent attacks of neutropenia with the interruption of chemotherapy. One of these children had three severe episodes of abdominal pains and mouth ulcers due to MTX therapy (resolved with leucovorin). Studies on impaired MTX elimination, an escalation of the leucovorin rescue, or enzymatic cleavage of the drug by carboxypeptidase in selected cases have succeeded in almost eliminating fatal toxicities [27].

Findings demonstrating specific serum IgG and IgA production in patients infected with *Cryptosporidium*, both HIV sero-positive and sero-negative, as compared to uninfected subjects [28], suggest the induction of a *Cryptosporidium*-specific humoral immune response in infected subjects. However, in this study, there was no difference in the number of patients with a positive response in the HIV-seropositive or sero-negative groups, indicating that HIV status may not play a significant role in the modulation of *Cryptosporidium*-specific antibody responses. The number of patients with positive IgG, IgM and IgA responses was also not significantly different in patients with or without a history of diarrhea, thereby indicating that *Cryptosporidium*-specific antibody responses may not necessarily be associated with protection from symptomatology [28].

Of the six ALL children with *Cryptosporidium* diarrhea, five were treated with an antimicrobial agent. The two sero-negative children were difficult to treat. *Cryptosporidium* diarrhea is self-limiting in immune-competent patients who do not require specific treatment. In immune-compromised patients, however, it is more severe and may be fatal. Various drugs have been tested for the treatment of *Cryptosporidium* diarrhea in these patients [29]. This severe, prolonged, and difficult-to-treat form of diarrhea was recognized in our study in ALL children with *Cryptosporidium* diarrhea.

In conclusion, children with ALL are more likely to suffer from severe gastrointestinal manifestations due to the disease itself or infectious and non-infectious complications of chemotherapeutic treatment (especially MTX and 6 MP). Diarrhea in ALL children is difficult to diagnose and treat. *Cryptosporidium* infection should be considered in cases of severe or prolonged watery diarrhea in ALL children. Since *Cryptosporidium* is not tested for in a routine stool examination, the laboratory should be notified for diagnostic confirmation using either a MZN stool stain or a more specific diagnostic tool, such as PCR.

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