Protozoal Colonization of the Intestinal Tract in Institutionalized Romanian Children

Darin K. Brannan, Ronald A. Greenfield, Willis L. Owen, David F. Welch, and Thomas L. Kuhls

From the Departments of Pediatrics, Medicine, and Biostatistics and Epidemiology, University of Oklahoma Health Sciences Center; and the Department of Veterans Affairs Medical Center, Oklahoma City, Oklahoma

To determine the prevalence of intestinal parasitic infections in 92 Romanian children institutionalized at Colentina Hospital (Bucharest, Romania) and at the Dystrophic Center (Vidra, Romania), medical charts were reviewed and complete physical examinations were performed. The nutritional status of each child was evaluated, and their sera were tested for the presence of antibodies to human immunodeficiency virus (HIV) and Cryptosporidium. Fecal samples were collected in 10% formalin and examined by an immunofluorescent assay and by trichrome staining for intestinal parasites. At least one protozoan was identified in 77% of the fecal specimens examined. Giardia lamblia (72% of cases), Cryptosporidium parvum (12%), and Entamoeba coli (4%) were the only parasites identified. Stepwise logistic regression revealed that the only factors predictive of giardia colonization were normal nutritional status ($P < .01$) and HIV seropositivity ($P < .02$), while cryptosporidium colonization was only associated with where the children lived ($P < .01$). Seventy-three percent of the children had IgA and/or IgG antibodies to Cryptosporidium in their sera. The presence of these antibodies was strongly associated with the severity of symptoms present in the HIV-infected children ($P < .01$). Protozoal colonization of the intestinal tract is common in institutionalized Romanian children and may play a role in causing morbidity and mortality in this high-risk group of children.

In 1989 it was discovered that >1,000 institutionalized children in Romania had AIDS [1, 2]. There has recently been a concentrated effort to halt the spread of this disease and to provide care to those who are HIV-seropositive. The prevalence of other infections in institutionalized Romanian children is generally unknown [3]. The purpose of this study was to define the prevalence of intestinal parasitic infections in this high-risk pediatric population.

Materials and Methods

Study Population

The study population included nonselected children residing in the AIDS Pavilion at Colentina Hospital (Bucharest, Romania) and at the Dystrophic Center (Vidra, Romania). Colentina Hospital is the center for the treatment of infectious diseases in Romania, while the Dystrophic Center is a facility for malnourished children. It is also a transitional facility between hospital and home for institutionalized children.

In July 1991 medical charts of the children were reviewed, and information regarding recent and current illnesses, the medications that they were receiving, and their CD4$^+$ lymphocyte counts measured within the preceding 6 months was recorded. Complete physical examinations were performed, and blood and fecal specimens were collected from each child. Diarrhea was defined as loose or watery stools that had been present for at least 24 hours and was present when the specimen was collected. All laboratory studies were performed at the University of Oklahoma Health Sciences Center (Oklahoma City).

This study was conducted with the approval of the University of Oklahoma Health Sciences Center Institutional Review Board and the Romanian Ministry of Health.

Determination of Nutritional Status

A growth chart was constructed with use of the sex- and age-specific 50th percentile for height and weight based on a survey of Romanian children [4]. The percent of estimated body weight for age and sex was calculated for each child and used as the criteria for determining the level of malnutrition. No malnutrition, moderately malnourished, and severely malnourished were defined as >65%, 51–65%, and ≤50% of the expected measurement for age, respectively [5].

Blood was collected in nonheparinized vials by means of an aseptic technique, and serum was stored at −70°C until it was tested. Serum albumin levels were measured with a Kodak Ektachem-700 (Kodak, Rochester, NY) by standard methodology. Serum transferrin levels were measured by an immunoas-
say with the Atlantic Antibodies kit (INCSTAR, Stillwater, MN) according to the manufacturer’s instructions. Serum albumin levels of ≤3.3 mg/dL and serum transferrin levels of ≤200 mg/dL were considered abnormal.

**HIV Analysis**

Antibodies to HIV type 1 were detected in the sera by ELISA (Kirkegaard and Perry, Gaithersburg, MD) and by western blot analysis (Organon Teknika, Durham, NC). An immunoblot was considered positive for HIV if gp160, gp120, and gp41; gp160, gp120, and p24; or p24 and gp41 were present as defined by the Centers for Disease Control and Prevention (CDC) [6]. HIV-seropositive children were classified according to the CDC pediatric classification system on the basis of medical chart reviews and physical examination results [7].

**Identification of Intestinal Protozoa**

Fecal specimens were collected in screw cap vials containing 15 mL of 10% formalin. All fecal specimens were concentrated by means of a parasite concentration system (Evergreen, Los Angeles) and a modified formalin–ethyl acetate method [8]. *Giardia lamblia* and/or *Cryptosporidium parvum* was detected in the fecal samples by an immunofluorescent assay (Meridian Laboratories, Cincinnati) [9]. In addition, trichrome-stained smears of fecal concentrates were examined by an experienced clinical parasitologist.

**Serology for Cryptosporidium Species**

The ELISA used to detect IgA and IgG antibodies to *Cryptosporidium* in the sera was adapted from a previously described method [10]. Following acquisition and purification of the *Cryptosporidium* oocysts, they were resuspended in PBS (pH 7.2) to a concentration of 5 × 10⁷ oocysts/mL, sonicated until >95% of the oocysts were disrupted, and frozen at −70°C until they were used in the assays. One hundred microliters of 5 × 10⁶ sonicated oocysts/mL in carbonate buffer (pH 9.6) was placed in each well of a 96-well ELISA plate (Nunc, Waperville, IL), and the plate was incubated overnight at 4°C. Following blocking with 1% bovine serum albumin, a 1:100 dilution of serum sample or control sample was added to each well, and the plate was incubated at 37°C for 1 hour. Following washing with 0.05% Tween 20 in PBS, 40 μL of a 1:150 dilution of alkaline phosphatase–conjugated goat antibodies to human IgA or IgG (Sigma, St. Louis) was added to each well, and the plate was again incubated. Color was developed by adding 100 μL of p-nitrophenyl phosphate (0.4 mg/mL; Sigma) in 10% diethanolamine dissolved in PBS to each well. The amount of substrate hydrolysis was measured with an automated microplate reader (Bio-Tek, Winooski, VT) as the optical density (OD) at 405 nm.

On each IgA or IgG ELISA plate, two positive control serum samples from individuals convalescing from cryptosporidiosis and three negative control serum samples from 6- to 12-month-old infants that consistently yielded low absorbance readings were tested (1:100 dilution) in duplicate. For the assay to be valid, the positive controls had to have mean ODs that were 2 SDs greater than the mean ODs of the negative controls. The sera were tested in duplicate on separate days. A specimen was considered IgA- or IgG-seropositive if the mean OD was greater than or equal to the (mean + 2 SDs) OD of the appropriate IgA- or IgG-negative controls.

**Statistical Analyses**

All statistical analyses were performed with SAS statistical software (SAS Institute, Cary, NC). Differences between means were compared by the Student’s t test. Differences in proportions were analyzed by likelihood ratio χ² tests or Fisher’s exact tests when appropriate for small sample sizes; a P value of < .05 demonstrated by two-tailed tests was considered significant. Stepwise logistic regression was performed with various dependent variables modeled against the following independent variables: place of residence; absent or present diarrhea; no, moderate, or severe malnutrition; normal or low serum albumin levels; normal or low serum transferrin levels; seronegative or seropositive HIV serological status; CDC classification of HIV-seronegative, HIV-seropositive and mildly symptomatic, HIV-seropositive and moderately symptomatic, or HIV-seropositive and severely symptomatic; and ≥1,000 or <1,000 CD4⁺ lymphocytes/mm³. Independent variables retained in the model at a P value of < .05 were reported as significant.

**Results**

Sixty children from Colentina Hospital and 32 children from the Dystrophic Center were evaluated. In both institutions 62% of the participants were male, and the children’s ages ranged from 12 to 52 months. However, the mean age of the participants at Colentina Hospital was 32 months compared with that of 25 months at the Dystrophic Center (P < .01).

All 60 children at Colentina Hospital were HIV-seropositive, while 13 children (41%) at the Dystrophic Center were HIV-seropositive. Immunoblotting confirmed the HIV seropositivity of all children tested by ELISA. At Colentina Hospital 5 children (8%) were mildly symptomatic by the CDC criteria for classifying pediatric HIV infection, 34 (57%) were moderately symptomatic, and 21 (35%) were severely symptomatic. At the Dystrophic Center two HIV-seropositive children were moderately symptomatic, and the remaining 11 HIV-seropositive children were severely symptomatic. At Colentina Hospital 47 children (78%) had CD4⁺ lymphocyte counts of ≥1,000/mm³, while 13 (22%) had CD4⁺ lymphocyte counts of <1,000/mm³.
CD4⁺ lymphocyte counts were not available for children at the Dystrophic Center.

Twenty-seven children (29%) had diarrhea at the time of observation. Eleven children (18%) at Colentina Hospital had diarrhea compared with 16 (50%) at the Dystrophic Center (P < .01). Diarrhea at the Dystrophic Center was more common in HIV-infected children (100%) than in HIV-seronegative children (18%) (P < .01). The prevalence of diarrhea in HIV-infected individuals was strongly associated with the CDC criteria for classifying pediatric HIV infection: no mildly symptomatic children, 22% among moderately symptomatic children, and 50% among severely symptomatic children (P < .01). Diarrhea was twice as common in children with CD4⁺ lymphocyte counts of <1,000/mm³ (31%) than in those with CD4⁺ lymphocyte counts of ≥1,000/mm³ (15%), but the difference was not statistically significant (P = .23).

According to growth parameters, 24 children (26%) were moderately malnourished and 11 (12%) were severely malnourished. Ten children had low serum albumin and/or transferrin levels; 5 children had low serum albumin and transferrin levels, 3 had low serum albumin levels only, and 2 had low serum transferrin levels only. Four (50%) of eight children with low serum albumin levels were severely malnourished, as were four (57%) of seven with low serum transferrin levels. Low levels of serum albumin (P = .02) and serum transferrin (P < .01) were significantly associated with malnutrition by growth parameters and with one another (P < .01). There were no significant differences in the distributions of malnutrition, low serum albumin levels, or low serum transferrin levels by center.

However, malnutrition was associated with diarrhea: 19% of children with normal nutritional status had diarrhea, 29% of children with moderate malnutrition had diarrhea, and 82% of children with severe malnutrition had diarrhea (P < .01). Similarly, the degree of malnutrition highly correlated with the CDC criteria for classifying pediatric HIV infection: none of the mildly symptomatic children were malnourished, 22% of the moderately symptomatic children were moderately malnourished, and 59% of the severely symptomatic children were moderately or severely malnourished (P < .01).

G. lambia cysts, C. parvum oocytes, and/or Entamoeba coli cysts were found in the fecal samples from 71 children (77%). E. coli was found in the stool specimens from only one child at Colentina Hospital and three children at the Dystrophic Center; therefore, no further analyses of colonization with this nonpathogenic protozoan were performed. No other intestinal protozoa or helminths were observed in the fecal specimens.

G. lambia was detected in fecal samples from 66 children (72%). Children at Colentina Hospital were more commonly colonized with Giardia than were children at the Dystrophic Center (P = .02; table 1). Other univariate associations with G. lambia colonization included absence of diarrhea, absence of malnutrition, normal serum albumin level, HIV seropositivity, and mild symptoms in HIV-infected children (table 1). However, stepwise logistic regression revealed that the only factors predictive of G. lambia colonization were normal nutritional status (P < .01) and HIV seropositivity (P = .02).

Cryptosporidium oocysts were detected in 11 fecal specimens (18%) from children at Colentina Hospital compared with no specimens from children at the Dystrophic Center (P < .01). Univariate or multivariate analyses did not demonstrate any other significant associations between C. parvum colonization and the variables outlined in table 1. C. parvum colonization was not significantly associated with G. lambia colonization (P = .72).

IgA and IgG antibodies to Cryptosporidium were much more commonly found in serum samples from children than were C. parvum oocytes in their fecal specimens (table 2). At Colentina Hospital, 42 children (70%) had IgA antibodies to Cryptosporidium, 35 (58%) had IgG antibodies to Cryptosporidium, and 44 (73%) had IgA or IgG antibodies to Cryptosporidium. At the Dystrophic Center, 20 children (63%) had IgA antibodies to Cryptosporidium, 16 (50%) had IgG antibodies to Cryptosporidium, and 23 (72%) had IgA or IgG antibodies.
Table 2. Univariate associations of place, acute diarrhea, nutritional status, and severity of HIV infection with the presence of IgA and IgG antibodies to Cryptosporidium in serum samples from institutionalized children in Romania.

<table>
<thead>
<tr>
<th>Variable (no. tested)</th>
<th>IgA</th>
<th>IgG</th>
<th>IgA and/or IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Place</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colentina Hospital, Bucharest (60)</td>
<td>70 (.47)</td>
<td>58 (.44)</td>
<td>73 (.88)</td>
</tr>
<tr>
<td>Dystrophic Center, Vidra (32)</td>
<td>63</td>
<td>50</td>
<td>72</td>
</tr>
<tr>
<td>Diarrhea</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent (65)</td>
<td>63 (.16)</td>
<td>58 (.37)</td>
<td>69 (.22)</td>
</tr>
<tr>
<td>Present (27)</td>
<td>78</td>
<td>48</td>
<td>81</td>
</tr>
<tr>
<td>Malnutrition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None (57)</td>
<td>58 (.04)</td>
<td>46 (.03)</td>
<td>65 (.07)</td>
</tr>
<tr>
<td>Moderate (24)</td>
<td>83</td>
<td>67</td>
<td>83</td>
</tr>
<tr>
<td>Severe (11)</td>
<td>82</td>
<td>82</td>
<td>91</td>
</tr>
<tr>
<td>Serum albumin level</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal (84)</td>
<td>69 (.43)</td>
<td>57 (.46)</td>
<td>74 (.68)</td>
</tr>
<tr>
<td>Low (8)</td>
<td>50</td>
<td>38</td>
<td>63</td>
</tr>
<tr>
<td>Serum transferrin level</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal (85)</td>
<td>68 (.68)</td>
<td>54 (.46)</td>
<td>72 (.67)</td>
</tr>
<tr>
<td>Low (7)</td>
<td>57</td>
<td>71</td>
<td>86</td>
</tr>
<tr>
<td>HIV serological status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative (19)</td>
<td>37 (.002)</td>
<td>42 (.19)</td>
<td>53 (.03)</td>
</tr>
<tr>
<td>Positive (73)</td>
<td>75</td>
<td>59</td>
<td>78</td>
</tr>
<tr>
<td>Symptom score of HIV-infected children1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild (5)</td>
<td>40 (.10)</td>
<td>40 (.12)</td>
<td>40 (.07)</td>
</tr>
<tr>
<td>Moderate (36)</td>
<td>72</td>
<td>59</td>
<td>75</td>
</tr>
<tr>
<td>Severe (32)</td>
<td>84</td>
<td>72</td>
<td>88</td>
</tr>
<tr>
<td>CD4+ lymphocyte count1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥1,000/mm³ (47)</td>
<td>74 (.16)</td>
<td>62 (.32)</td>
<td>79 (.09)</td>
</tr>
<tr>
<td>&lt;1,000/mm³ (13)</td>
<td>54</td>
<td>46</td>
<td>54</td>
</tr>
</tbody>
</table>

* Analyzed by likelihood ratio χ² tests or Fisher’s exact tests when appropriate.
1 According to the Centers for Disease Control and Prevention’s criteria for classifying pediatric HIV infection [7].

Discussion

Before 1989, >1,000 institutionalized children in Romania had AIDS because of the common practice of giving multiple therapeutic transfusions of unscreened blood to malnourished children and the frequent use of improperly sterilized needles and syringes [1, 2]. Nearly all of the initially described children with AIDS had ≥10% loss of body weight, and 56% of the children had chronic diarrhea for >1 month in duration [1]. Since gastrointestinal symptoms were common in this pediatric population, fecal samples from institutionalized children at two sites in Romania where HIV-infected patients are commonly cared for were evaluated for the presence of intestinal parasites. In this study the presence of diarrhea and severity of malnutrition in the children highly correlated with the CDC criteria for classifying pediatric HIV infection [7].

G. lamblia and C. parvum were commonly observed in the fecal specimens from the children (72% and 12%, respectively), while E. coli was found in the stool specimens from four children. No other intestinal pathogens were identified; however, more delicate protozoa such as Dientamoeba fragilis may not have been detected because of the harsh concentration techniques that were utilized in the study. In addition, protozoa such as Cyclospora cayetanensis, Isospora belli, and the intestinal microsporidia would not have been detected by the examination techniques that were used.

G. lamblia and C. parvum can cause diarrheal disease or asymptomatic infection in both immunocompetent and immunocompromised individuals [11]. In this population of children, there was no association between acute diarrhea at the time the specimen was collected and the presence of Giardia or Cryptosporidium in the feces. In fact, univariate analysis showed that detection of G. lamblia in the feces was associated with the absence of diarrhea. However, only one fecal specimen from each child was examined, and the volume of stool in specimens from children with diarreal stools may have been substantially less than that in samples from children with formed stools; thus, many of the children with acute diarrhea for whom the fecal examination was negative may have actually had giardiasis. An association was also identified between worsening nutritional status of the children and the failure to detect G. lamblia in the children’s feces. In a previous study of
Israeli children attending a day-care center, giardia colonization was also associated with improved growth parameters [12]. Seventy-nine percent of the children evaluated in this study were HIV-seropositive. Most of the HIV-infected children were classified as moderately or severely symptomatic on the basis of the CDC classification system, though by immunologic criteria only 22% of the children at Colentina Hospital had recent CD4⁺ lymphocyte counts of <1,000/mm³. G. lamblia colonization was associated with HIV seropositivity, although more severely malnourished HIV-infected children were less likely to have G. lamblia in their feces than were mildly symptomatic HIV-infected patients.

Cryptosporidium oocysts were identified in fecal samples from 18% of HIV-infected children at Colentina Hospital. However, univariate and multivariate analyses did not identify HIV seropositivity as a risk factor for cryptosporidiosis since no children at the Dystrophic Center had cryptosporidiosis. Many individuals may excrete oocysts in concentrations too low to be detected by routine fecal examination techniques, though the immunofluorescent assay used in this study is the most sensitive method available for detecting fecal oocysts [9].

The detection of antibodies to Cryptosporidium in the serum has been used to better diagnose infection and to define the epidemiology of cryptosporidiosis, even in patients with AIDS [10, 13-22]. In this study 73% of the children had IgA and/or IgG antibodies to Cryptosporidium in their sera. Similar to the findings of other studies, 17% of children had IgA antibodies to Cryptosporidium in their sera without C. parvum-specific IgG [14, 21]. The levels of antibodies to Cryptosporidium (IgA, IgG, and IgM) have been shown to be higher in the sera from HIV-infected patients with chronic cryptosporidiosis than in the sera from HIV-infected patients without cryptosporidiosis and HIV-seronegative individuals; thus, it is unlikely that the presence of C. parvum-specific antibodies results only from polyclonal B cell activation [21].

Multivariate analyses revealed that the presence of serum IgA antibodies to Cryptosporidium was highly associated with HIV seropositivity and worsening symptom scores in Romanian HIV-infected children, while C. parvum-specific IgG was also associated with severity of malnutrition, absence of diarrhea, and absence of hypoalbuminemia. Protein-losing enteropathy with diarrhea and a low serum albumin level may have been responsible for the lower level of serum IgG antibodies to Cryptosporidium in some of the more severely affected children.

Giardia and Cryptosporidium are frequently found in the stools from noninstitutionalized Romanian children. In 1976 G. lamblia was detected in fecal specimens from 13% of 9,316 Romanian children in an urban district [23]. Similar to the findings in this study, Giardia was more commonly found in the fecal samples from asymptomatic children than in those from symptomatic children. In 1991 Cryptosporidium oocysts were identified in stools from 3% of 657 children hospitalized because of acute diarrhea at two large pediatric units in Romania [24]. Crowding, poor sanitary conditions, and a high proportion of malnourished and immunocompromised patients may have played a role in the higher rates of protozoal colonization that were observed in this study. High prevalence rates of giardia and cryptosporidium colonization have also been identified among other groups of institutionalized children [17, 25]. However, nearly 80% of the children in this study were HIV-seropositive, and these children will become progressively more immunodeficient. Whether protozoal colonization plays a role in causing increased morbidity and mortality in this high-risk group of children requires further study. It has been previously recommended that symptomatic and asymptomatic children in institutions with high rates of giardia colonization should be treated and continually monitored for fecal excretion of Giardia [25]. There is currently no effective treatment for eradicating Cryptosporidium from the intestines [11]. Whether treating giardia infection, grouping of colonized individuals, or emphasizing personal and environmental hygiene would reduce the rate of giardia colonization in institutionalized Romanian children is unknown.

Acknowledgments

The authors thank Rose Stursa for her administrative assistance, David Crawford and David Horn for their technical assistance, and Dr. Gheorghe Jipa (Colentina Hospital of Infectious Diseases, Bucharest, Romania) and Dr. Nicolae Beldescu (Department of Preventive Medicine, Ministry of Health, Bucharest) for their assistance in coordinating the evaluation of the children.

References


