ENTEROCYTOZOON BIENEUSI AMONG CHILDREN WITH DIARRHEA ATTENDING MULAGO HOSPITAL IN UGANDA

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Abstract. The prevalence of Enterocytozoon bieneusi in the general population is unknown. Using genetic tools, we investigated its prevalence and contribution to diarrhea and malnutrition in hospitalized children in Uganda. A cross-sectional, case-control study involving diarrheic children who were matched for age and sex (3:1) with control children. Measurements included anthropometry and clinical assessment. A total of 17.4% of 1,779 children with diarrhea were infected with Enterocytozoon bieneusi compared with 16.8% of 667 control children ($\chi^2 = 0.137, P = 0.712$). Prevalence was highest during the rainy seasons. There was no significant relationship between infection with Enterocytozoon bieneusi and stunting, being underweight, wasting, or acute diarrhea. However, children who were Enterocytozoon bieneusi-positive by a polymerase chain reaction (PCR) had diarrhea for a longer period (15.15 versus 9.67 days; $F = 12.02; P = 0.001$) compared with children who were either uninfected or were Enterocytozoon bieneusi-positive by a nested PCR. We conclude that Enterocytozoon bieneusi is widespread among children 3–36 months of age in Uganda, and that in a cross-sectional study, there was no clear association of Enterocytozoon bieneusi with poor nutrition or diarrhea. Since Enterocytozoon bieneusi is closely linked with persistent diarrhea and wasting in adults who are positive for human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS), the outcome of follow-up studies involving children who are HIV/AIDS-positive and severely malnourished children may be entirely different and warrants further study.

INTRODUCTION

Enterocytozoon bieneusi was first described in 1985 in patients with acquired immunodeficiency syndrome (AIDS) and chronic diarrhea and wasting.1 Over the following decade and a half, many reports have confirmed the link of this microsporidium with wasting and chronic diarrhea, often involving hepatobiliary disease, in patients with AIDS throughout the world. Much of the information regarding microsporidiosis, in particular Enterocytozoon bieneusi, is critically and extensively reviewed in the literature,1–6 and is considered the most common intestinal infection associated with persistent diarrhea and wasting, occurring in up to 50% of patients with AIDS.5,6 While other intestinal microsporidiosis, such as Enterocytozoon intestinalis (formerly Septata intestinalis), may also contribute to diarrhea and wasting,7,8 the majority of cases are due to Enterocytozoon bieneusi.5,6,7,9–11 Intestinal microsporidiosis is associated with marked malabsorption of vitamins, micronutrients, carbohydrates, and fats. When compared with Enterocytozoon bieneusi-negative patients with persistent diarrhea, individuals with intestinal microsporidiosis had significantly increased fat malabsorption, decreased xylose absorption, decreased red blood cell folate, decreased vitamin B$_{12}$ absorption, and lower serum zinc levels.9–11 In addition, patients with microsporidiosis had lower mean Karnofsky scores.10 Small intestinal injury with partial villus atrophy and crypt hyperplasia was seen in half of the patients with microsporidia, but was also found in pathogen-negative controls with diarrhea.10–12 The high prevalence of intestinal microsporidiosis in persons infected with the human immunodeficiency virus (HIV) and its association with malabsorption contributes considerably to AIDS wasting.13 In the majority of these studies, the CD4 lymphocyte count in peripheral blood tends to be less than 100/mm$^3$.13,14

The literature from Africa is limited and the prevalence of this infection in children in most countries, including Uganda, is unknown.15,16 In studies from Zimbabwe15 and Guinea-Bissau,17 18% and 11% of patients infected with HIV and with chronic diarrhea, respectively, were positive for microsporidia. Whether Enterocytozoon bieneusi is a risk factor for persistent diarrhea and wasting in children who are HIV/AIDS-positive has not been investigated, nor has the contribution, if any, of the infection to diarrhea and malnutrition in children who are HIV/AIDS-negative. Several cases of Enterocytozoon bieneusi infection in HIV-negative patients with diarrhea have been reported only in adults.16,18

The objective of this study was to conduct a comprehensive and systematic cross-sectional, case-control investigation to 1) determine the prevalence of Enterocytozoon bieneusi in children in Uganda, and 2) determine whether Enterocytozoon bieneusi contributes to diarrhea and/or malnutrition in hospitalized children attending Mulago Hospital.

MATERIALS AND METHODS

Patients/study population. The study population included children 0–60 months of age attending the Mulago Hospital with diarrhea (persistent or acute). An episode of diarrhea was defined as being characterized by three or more loose motions after more than 72 hours from the last day of diarrhea.

Study design. This was a cross-sectional and case-control study in which every three children with diarrhea were matched for age and sex with a child without diarrhea.

Inclusion criteria. Children 0–60 months old attending the Mulago Hospital with acute or persistent diarrhea and whose caretakers consented to participate in the study were entered into the study from November 1999 to January 2001. For the controls, children without diarrhea or any other infection in the previous 72 hours were recruited from other hospital wards.

Exclusion criteria. Children with dysentery, measles, other infections, or any form of cancer were excluded from the study. Children whose parents or caretaker could not ascertain their age were also excluded from the study.

Sample size calculations. A formula of Kish was used to calculate the sample size.19 Assuming the prevalence of Enterocytozoon bieneusi to be 18%, and a margin of error of 1% and 95%...
confident intervals (CIs), a sample size of 2,202 was calculated for the prevalence of *E. bieneusi*. The sample size for the case-control study was calculated using the formula by Fleiss. With an 80% power, a 95% CI, and an odds ratio (OR) worth detecting at 1.52, the sample size of the affected population (children with diarrhea) was 1,599 and that of the controls (children without diarrhea) was 588. This was based on *E. bieneusi* as a risk factor for diarrhea. *Enterocytozoon bieneusi* was estimated to occur in 12.2% of the children with diarrhea and in only 8% of the controls.

**Recruitment.** Up to 15 eligible children were recruited daily. The children were selected by systematic sampling of every sixth child with diarrhea. Due to resource constraints, it was not possible to follow up all the children, or determine their HIV status. Using simple random sampling, we selected five of the 15 children daily for a follow-up while at the hospital. Children were assessed clinically and their nutritional status was determined. Children received appropriate fluid and/or antibiotic therapy as needed.

**Measurements.** Measurements were carried out using international guidelines. Children were weighed to the nearest 100 grams using a hanging Salter (Minneapolis, MN) spring scale, which was checked and adjusted daily. Children were weighed almost nude with only underpants retained for privacy. Height for children 24–59 months old and length for those less than two years old were measured using locally constructed adjustable wooden boards constructed according to specifications of the United Nations. Height was taken to the nearest 0.1 cm. Mid upper arm circumference was measured to the nearest 0.1 cm using a standard tape acquired for this purpose. The age of the child was ascertained from the child’s health card, baptismal or birth certificates and if none of these were available, age was assessed using a calendar of local events such as the 1996 general election.

**Stool collection and DNA analysis.** Stools were collected into disposable plastic containers and were refrigerated at 4°C. Fecal specimens were transported every month to Tufts University in the United States for DNA extraction and polymerase chain reaction (PCR) analysis, normally performed within two weeks after arrival. The PCR and sequence analysis were performed with the aim of detecting *E. bieneusi* and to obtain genotype information using the internal transcribed spacer (ITS) region sequence.

The DNA was extracted from fecal samples essentially as we described previously. The PCR amplification was performed using two different sets of nested primers specific for *E. bieneusi*. The first PCR amplification incorporated primers EBIER1 and EBIEF1 primers as described by da Silva and others, followed by a second amplification with nested primers EBIER6 (5′-GGTCATAGGGTAAAGTGC-3′) and EBIEF5 (5′-TCTTCCCTTGCAATTGC-3′). Cycling parameters for the first PCR were 35 cycles at 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 40 seconds. An aliquot (1 μL) of the first PCR product was used as template for the second nested reaction using 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 40 seconds as the cycling parameters. A 350-base pair control DNA template, amplified by the first set of primers, was included in each set of reactions to test for false-negative results due to inhibition of the PCR. Positive reactions were confirmed by a second set of nested PCRs that amplify the ITS region, as well as a portion of the flanking large and small subunit ribosomal RNA genes, which was subsequently used for sequence analysis. The ITS outer primers were EBIT3 (5′-GTTCATAGGGTAAAGTGC-3′) and EBIT4 (5′-TCTTCCCTTGCAATTGC-3′) with cycling parameters of 35 cycles at 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 40 seconds, and the inner primers were EBIT1 (5′-GCTTCAATTGC-3′) and EBIT2.4 (5′-ATCTTGAGCCGATCC-3′) with cycling parameters of 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 40 seconds. The PCR products were subjected to electrophoresis in a 1.5% agarose gel and visualized by staining the gel with ethidium bromide. Negative and positive controls were included in all sets of PCRs, as well as amplifying the negative control from the first PCR in the second reaction to check for low-level contamination. A mock sample containing no feces was also included for each set of DNA extractions to check for possible experimental contamination, as well as no DNA PCR controls.

The PCR products of the expected size identified stools containing *E. bieneusi*. Specificity for *E. bieneusi* was confirmed by digestion of the EBIT3/EBIT2.4 PCR products with Msp Al I restriction enzyme as we described previously. Some amplicons were randomly chosen for sequencing to further confirm the specificity of the assay and to obtain genotypic information on human isolates of *E. bieneusi* from the study area.

**Ethical considerations.** The study was approved by the Mulago Hospital Research and Ethics Committee. Relevant treatment was offered to the children and the attending pediatrician was advised of the stool examination results. Children were followed-up until discharged from the hospital. No follow-up in the community was done because of budgetary constraints. Since there is no readily available effective therapy for infection with *E. bieneusi*, children with this infection were offered symptomatic treatment, as well as relevant treatment of any concurrent infections.

**Data analysis.** Data was processed using the EpiBAS and Epi-Info version 6.0 computer statistical package (Centers for Disease Control and Prevention, Atlanta, GA) and SPSS software (SPSS, Inc., Chicago, IL). Data was summarized using frequency tables, bar charts, histograms, means, and standard deviations. Possible risk factors for *E. bieneusi* were analyzed as follows: for categorical risk factors, contingency tables were used and the strength of association was measured using the chi-square test and its associated P value.

For continuous risk factors the Student’s t-test was used to compare the mean value of the risk factors in the study and control children. To assess the interactions and joint effects of the risk factors, logistic regression analysis was used. Background sociodemographic data were collected using a questionnaire.

**RESULTS**

**Prevalence of *E. bieneusi*.** Overall, 310 (17.4%) of the 1,779 with diarrhea were infected with *E. bieneusi* compared with 112 (16.8%) of the 667 children without diarrhea. The difference between these two groups was not statistically significant ($\chi^2 = 0.137, P = 0.712$). The mean age of the children with diarrhea was 14.0 months, as shown in Figure 1. The age of children with diarrhea ranged from four to 60 months. There seemed to be an overlap between the age distribution
of children infected with *E. bieneusi* (Figure 2) and that of children with diarrhea.

**Diarrhea and *E. bieneusi***. Of the 532 children with persistent diarrhea, 100 (18.8%) were infected with *E. bieneusi* compared with 210 (16.8%) of the 1,247 children with acute diarrhea. The difference between these two groups was not statistically significant ($\chi^2 = 0.992$, $P = 0.319$). However, among the infected children, there was a significant relationship between the intensity of infection with *E. bieneusi* and the duration of diarrhea. Children with very intense infection as detected by PCR had diarrhea lasting an average of 15.15 days, compared with only 9.67 days among those in whom the detection required the nested PCR amplification. This difference was statistically significant ($F = 12.02$, $P = 0.001$). Thus, children who excreted more spores in their stool were more likely to have diarrhea that lasted longer than children who either excreted very few spores or were uninfected ($P < 0.001$, OR = 8.99, 95% CI = 5.55–14.0).

**Breast-feeding**. Of the 1,287 children who were still breast-feeding, 216 (16.8%) were infected with *E. bieneusi* compared with 206 (17.8%) among the 1,159 not breastfeeding ($\chi^2 = 0.419$, $P = 0.517$). However, only one (10%) of the 13 children who were reportedly exclusively breast-feeding was infected with *E. bieneusi*.

**Nutritional status**. **Wasting**. Of the 620 children with wasting (weight-for-height Z-score [WHZ]-score < -2 SD) and diarrhea, 107 (17.3%) were infected with *E. bieneusi* compared with 203 (17.5%) of the 1,159 children who were not wasted. This difference was not statistically significant ($\chi^2 = 0.02$, $P = 0.892$).

**Underweight**. Of the 917 underweight children with diarrhea, 167 (18.2%) were infected with *E. bieneusi*. Conversely, only 143 (16.6%) of the 862 children with normal weights and diarrhea were infected with *E. bieneusi*. The difference was not statistically significant ($\chi^2 = 0.81$, $P = 0.367$).

**Stunting**. Of the 641 stunted children with diarrhea, 117 (18.3%) were infected with *E. bieneusi* compared with 193 (17.0%) of the 1,138 diarrhea children who were not stunted. The difference was not statistically significant at the 95% CI ($\chi^2 = 0.48$, $P = 0.490$). Overall, the presence or intensity of infection with *E. bieneusi* did not significantly influence the nutritional status.

**Seasonal variation**. The monthly prevalence rates of infection with *E. bieneusi* are shown in Figure 3. The prevalence of infection with *E. bieneusi* correlated with the two major peaks for rainfall in Kampala, Uganda, which occurred during the months of the March-May and September-November.

**Enterocytozoon bieneusi and the prevalence of AIDS by the World Health Organization (WHO) clinical case definition**. According to the WHO modified clinical case definition for pediatric AIDS,24 suspected cases present with at least two major and two minor defined clinical signs in the absence of other known causes of immune suppression, such as cancer and severe malnutrition or other recognized etiology. The major signs include weight loss or abnormally slow growth (failure to thrive or a weight-for-age Z-score < -2 SD or a WHZ-score < -2 SD), chronic diarrhea (> 1 month), prolonged fever (> 1 month), and severe or repeated pneumonia. The minor signs include generalized lymphadenopathy (lymph nodes measuring at least 0.5 cm present in 2 or more sites, with bilateral nodes counting as one site), oro-
pharyngeal candidiasis, repeated common infections such as otitis, pharyngitis, and generalized pruritic dermatitis, and confirmed maternal HIV infection. Overall, 42 (1.7%) of the 2,446 study children had at least two major and two minor signs of AIDS or were known HIV/AIDS-infected children. Of the 42 children with suspected AIDS, 26 (61.9%) had diarrhea, 15 (35.7%) had persistent diarrhea, while 16 (38.1%) had no diarrhea. Only nine of the 42 children (21.4%) were infected with *E. bieneusi*.

**Outcome.** Information on outcome was available for 736 children with diarrhea.

**Mortality.** There was no significant difference between the mortality of those infected with *E. bieneusi* and those who were not infected (6.9% of 130 versus 8.1% of 606).

**Unfavorable outcome.** This was defined as either death or failure to respond to treatment. There was no significant difference between those infected with *E. bieneusi* (70%) and those who were not infected (66.5%) regarding the unfavorable outcome ($\chi^2 = 0.594, P = 0.441, OR = 1.053, 95% CI = 0.928–1.194$). Of the 11 children with diarrhea and meeting the WHO case definition criteria for AIDS, three (27.3%) died compared with 55 (7.6%) of the 725 children not meeting the WHO criteria for AIDS.

**Gene sequence analysis.** We have directly sequenced the PCR products amplified with the EBITSI/EBITTS2.4 primer pair from 10 randomly selected samples that were positive for *E. bieneusi* by PCR analysis. The sequences of these 350-base pair fragments were compared with sequences in the GenBank database by BLAST analysis. All 10 sequences had highest sequence identity to *E. bieneusi* sequences in GenBank; thus, confirming the specificity of the *E. bieneusi* PCR assay. Six of the Ugandan sequences were identical to the *E. bieneusi* genotype K small subunit ribosomal RNA gene/ITS/large subunit ribosomal RNA gene sequences, which were isolated from a cat (GenBank accession number AF267141, nucleotides 50-399). The majority of this sequence spans the ITS region (nucleotides 107-349). The remaining four sequences had highest sequence identity (99.4%) to the *E. bieneusi* genotype K sequence, but differed by two bases. Interestingly, one of these base differences (T → C; nucleotide 92) was observed in only one other *E. bieneusi* sequence (AF348475, 99.4% identity) isolated from swine, and the second base difference (G → A; nucleotide 88) was found in approximately 40% of the *E. bieneusi* sequences, including AF348475. Of the 10 randomly selected samples, five were single-round amplification products and the remaining five represented nested PCR products. There was no correlation between the single and nested PCRs and which of the two sequences was isolated.

The nucleotide sequence of isolate UG2145 (accession number AF502396), which is representative of the non-genotype K sequence, has been submitted to GenBank.

**DISCUSSION**

This is the first comprehensive and systematic study of infection with *E. bieneusi* in children anywhere, including Uganda. The prevalence of infection with *E. bieneusi* (approximately 17%) among children 6–36 months old was surprisingly high. In this cross-sectional study, there was no clear association between infection and diarrhea or malnutrition.

The high prevalence of infection with *E. bieneusi* in children and the lack of apparent association with diarrheal illness were unexpected. This may have been due to the design of the study. In follow-up studies of children who were either HIV/AIDS-positive or with severe malnutrition, the outcome might have been different. This view is based on numerous observations in HIV/AIDS-positive adult patients in whom infection with *E. bieneusi* is strongly linked with chronic diarrhea and wasting.\(^1\)–\(^6\) Due to budgetary constraints, we were unable to determine the HIV status of the children in this study, which is estimated to be 18–20% among these children (Tumwine JK, unpublished data). The observation that children with a high rate of excretion of spores were more likely to have diarrhea of longer duration ($P < 0.001$) is curious, and may suggest an association with more prolonged illness. Intestinal *E. bieneusi* has been linked to marked malabsorption of vitamins, micronutrients, carbohydrates and fats.\(^9\)–\(^11\)

The overlap between the age distribution of diarrhea and infection with *E. bieneusi* suggests that a high proportion of the children in the 3–36-month-old age group acquire the infection for the first time, as they do with other highly prevalent enteric infections, including cryptosporidiosis (Tumwine JK and Tzipori S, unpublished data). It was significant that the infection is self-limiting and does not appear to become persistent in children after exposure, since the number of children excreting spores decreases after 36 months of age (Figure 1). This means that like other infections, children and adults are regularly exposed to *E. bieneusi* and that in the absence of immune abnormalities, the infection probably is self-limiting and asymptomatic. The situation is different when infection occurs in individuals who are immunocompromised, which presumably include HIV-AIDS-positive children, and possibly children with severe malnutrition. Poorly nourished children also tend to have immune abnormalities, although these are not as well-defined as they are for children with AIDS. While there was no evidence that breast-feeding accorded the children protection against infection with *E. bieneusi* in the current study, only one of the 13 exclusively breast-fed children were infected. There was a clear seasonal variation in the detection rate of infection with *E. bieneusi*, with the highest prevalence recorded in the rainy season.

Identification of *E. bieneusi*-positive stool samples was determined by a nested PCR using *E. bieneusi*-specific primers.\(^22\),\(^23\) Positive samples were confirmed by a second nested PCR, of which 10 PCR products were randomly sequenced to verify the specificity of the assay. All 10 sequences had highest sequence identity to *E. bieneusi* sequences, with the majority being identical to isolates of other mammalian species rather than the published human isolates. Since some of the sequences were not unique, it suggests no correlation with geographic location or host species.

In summary, *E. bieneusi* is a highly prevalent infection among children 3–36 months of age. While there appears to be no association between infection and either diarrhea or poor nutrition, children who were excreting more spores tended to have diarrhea of longer duration than children who were either excreting very low numbers or were apparently uninfected. The contribution of *E. bieneusi* to wasting and persistent diarrhea in HIV-AIDS-positive children and in the severely malnourished warrants further study.

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