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## CRYPTOSPORIDIOSIS AND MICROSPORIDIOSIS IN UGANDAN CHILDREN WITH PERSISTENT DIARRHEA WITH AND WITHOUT CONCURRENT INFECTION WITH THE HUMAN IMMUNODEFICIENCY VIRUS

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**Abstract.** *Cryptosporidium* spp. and *Enterocytozoon bienersi* are enteric pathogens that have emerged as significant causes of persistent diarrhea (PD) in immunologically compromised individuals particularly in association with HIV/AIDS. We conducted a cross-sectional study on the clinical epidemiology of *E. bienersi* and *Cryptosporidium* in children with PD, with and without HIV/AIDS, attending Uganda's Mulago National Referral Hospital. Two hundred forty-three children aged < 60 months, admitted between November 2002 and May 2003 with PD (> 14 days), were analyzed for HIV status and CD4 lymphocyte counts, and stools were screened for the presence of *E. bienersi* and *Cryptosporidium* by microscopy and positive samples genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. Eighty (32.9%) of the children were excreting *E. bienersi*, and 76 (31.3%) were excreting *Cryptosporidium*. Ninety-one of the 243 children had HIV, of who 70 (76.9%) had *E. bienersi*, versus 10 (6.6%) of the 152 without (odds ratio = 47.33; 95% CI = 19.88 to 115.97), while 67 (73.6%) had *Cryptosporidium*, versus 9 (5.9%) without (odds ratio = 44.36; 95% CI = 18.39 to 110.40). Children with counts < 25% CD4 cells were more likely to have either *E. bienersi* (odds ratio = 7.42; 95% CI = 3.77 to 14.69) or *Cryptosporidium* (odds ratio = 6.45; 95% CI = 3.28 to 12.76) than those with higher CD4 percentages. However, only HIV status was independently associated with either *Cryptosporidium* or *E. bienersi*. Among the 243 children with PD, 67 (27.8%) were infected with both enteric pathogens, with HIV being the only independent predictor of coinfection. Finally, some 81% of HIV-infected children with PD excreted one or both organisms, compared with only 10% of children with PD testing negative for HIV. Seventy-four percent of isolates were *C. hominis*, the anthroponotic species, 17% were *C. parvum*, the zoonotic species, and 8% were a mixture of the two or others.

### INTRODUCTION

Cryptosporidiosis and microsporidiosis are two enteric microorganisms most commonly associated with persistent diarrhea (PD) and wasting in immunologically compromised individuals involving mostly people with HIV/AIDS, and against which there is no effective therapy.<sup>1–4</sup> Whereas cryptosporidiosis is known to also be a common serious cause of acute diarrhea in all ages of immunologically healthy people,<sup>5,6</sup> microsporidiosis, as judged by the lack of scientific documentation, does not appear to be associated with neither acute illness, nor, it seems in general, with immunologically healthy people. Indeed, *Enterocytozoon bienersi*, the microsporidia most commonly associated with PD and wasting in people with HIV/AIDS, was unknown prior to the emergence of AIDS in the mid-1980s.<sup>7</sup> Until the introduction of antiretroviral therapy (ART) in 1995–1996,<sup>3</sup> both were serious opportunistic infections that complicated HIV/AIDS in developed countries. In the absence of ART, adults in developing countries, unfortunately, continue to suffer the consequences of cryptosporidiosis and microsporidiosis of profound PD and wasting in patients with HIV/AIDS.<sup>1–3,8–10</sup> Although there are several reports from Africa on cryptosporidiosis in children,<sup>11–16</sup> there is little on microsporidiosis. Furthermore, most of the worldwide reports, with a few exceptions,<sup>17,18</sup> tend to focus on adults with HIV/AIDS.<sup>19–21</sup>

In previous communications, we have shown that cryptosporidiosis<sup>22</sup> and microsporidiosis<sup>23</sup> are very common infections in infants and children in Uganda aged < 60

months. In these studies, while cryptosporidiosis was shown to be closely linked with acute or persistent diarrhea wasting and poor prognosis in some 25% of 1,779 children investigated, there was no evidence that *E. bienersi*, observed in approximately 17% in the same population, was clearly associated with illness.<sup>23</sup> The role of HIV/AIDS in these children however was not determined, which may explain the lack of apparent clinical significance of *E. bienersi* in the overall study population of some 1,779 children.<sup>23</sup> Consequently, an additional study was undertaken to specifically determine the contribution of cryptosporidiosis and microsporidiosis to pediatric PD, with and without HIV/AIDS.

### MATERIALS AND METHODS

**Study design, site, and population.** Children aged less than 60 months, admitted to Mulago Hospital with PD (> 14 days) and whose caretakers consented to participate in the study, were recruited from November to 2002 to May 2003 (Table 1). None of the patients were on ART on recruitment. Children with measles, dysentery, or cancer were excluded from the study.

This was a cross-sectional study in which we determined the prevalence, by HIV status, of *E. bienersi* and *Cryptosporidium* among children attending Mulago Hospital with PD. The study was carried out in the pediatric diarrhea treatment ward of Mulago Hospital, in Kampala. Mulago Hospital is the largest referral and teaching hospital in the country. On average, 40 to 60 patients are admitted each day, of these, at least 5 have PD. Patients aged less than 60 months are admitted to the diarrhea treatment ward. After explaining the purpose of the study to the parents/caretakers and getting informed consent, patients fulfilling inclusion criteria were enrolled into the study consecutively until the sample size was reached.

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TABLE 1  
Features of children with persistent diarrhea by HIV status

Feature	HIV-positive (N = 91)	HIV-negative (N = 152)	P value
Mean age in months (SD)	14.38 (9.43)	11.45 (5.96)	0.030
Median CD8+ cell count/ $\mu$ L (IQR)	586.5 (0–1643.8)	514.0 (0–892.0)	< 0.01
CD4+ cell % (SD)	18.31 (11.49)	33.82 (13.27)	< 0.01
Wasted (%) <sup>*</sup>	20 (22.0)	20 (13.2)	0.054
Underweight (%) <sup>†</sup>	43 (47.3)	55 (36.2)	0.059
Stunted (%) <sup>‡</sup>	57 (62.6)	76 (50.0)	0.037

IQR, Interquartile range.

<sup>\*</sup> Weight for height z-score  $\leq -2$ .

<sup>†</sup> Weight for age z-score  $\leq -2$ .

<sup>‡</sup> Height for age z-score  $\leq -2$ .

Stools were collected from the diapers or obtained directly and placed into plastic containers, using disposable gloves. The stools were taken daily to the Joint Clinical Research Center (JCRC) in Kampala, for storage at 4°C, after which they were shipped unfrozen in batches to Tufts University, North Grafton, MA. The stools were tested for *E. bieneusi* by PCR,<sup>23</sup> and for *Cryptosporidium* by immunofluorescence microscopy using specific *C. parvum* antibodies,<sup>2</sup> and subsequently confirmed and genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis.<sup>22</sup> HIV tests and CD4+ lymphocyte counts were carried out at the Uganda Virus Research Institute. Blood was transported at ambient temperature and CD4 cell counts were performed within 12 hours. The CD4+ lymphocyte count was done using dual color flow cytometry (FACScan instrument and MultiSet software, Becton Dickinson, Franklin Lakes, NJ). The HIV status was determined using two enzyme linked immunoassays (Recombigen HIV-2, Trinity Biotech plc, Bray CC, Wicklow, Ireland; and Wellcozyme HIV Recombinant, Murex Biotech Ltd., Dartford, UK), and Western blots as well where necessary. For children aged below 18 months who had a positive HIV test by ELISA, their status was confirmed by reverse transcriptase PCR.

**Sample size and statistical tests.** To estimate the prevalence of *E. bieneusi*, a sample size of 211 children was calculated using a formula by Kish and Leslie.<sup>24</sup> This was based on an estimated prevalence of 18.8% of *E. bieneusi* among a population of 2,140 children seen in the clinic over a period of 7 months, and allowing for a 5% error and 95% confidence intervals (CI).

Data were entered into EPIINFO version 6 (Centers for Disease Control and Prevention, Atlanta, GA) and analyzed using SPSS. Statistical significance differences between CD4 cell counts by *E. bieneusi* or *Cryptosporidium* status were determined using Mann-Whitney *U* nonparametric tests. For normally distributed data, the Student *t* test was used to compare differences in mean values. Categorical variables were compared using the  $\chi^2$  test or Fisher exact test where the number in any cell was five or less; with the corresponding odds ratios, 95% confidence intervals and *P* values. A *P* value of less than 0.05 was considered significant. Logistic regression analysis was used to establish factors independently associated with coinfection of the two organisms.

**Ethical issues.** The study was reviewed and approved by the Makerere University Medical School and Mulago Hospital Research and Ethics Committee. All interviews were conducted in the local language (usually *Luganda* or English, which are widely spoken) with a translator, where necessary, and consent forms were translated into the relevant local lan-

guage and the study physician obtained informed consent. Parents or guardians were offered pre- and post-HIV counseling; and relevant treatment was offered to the children and the results were given to the attending physician as soon as they were available. Children testing positive for HIV were referred to the Mulago Hospital Pediatric Infectious Disease Clinic for follow up and further management.

## RESULTS

Two hundred forty-three children with PD were recruited, of who 145 (59%) were males, with an age range of 1–60 months (mean = 13.0; SD = 8.67). Overall, 80 (32.9%) of the 243 children with PD were excreting *E. bieneusi* in the stool, and 76 (31.3%) were excreting *Cryptosporidium* oocysts. In contrast, in previous studies the rate of excretion of *Cryptosporidium* oocysts among 667 healthy children was 8.5%<sup>22</sup> and of *E. bieneusi* spores 16.8%.<sup>23</sup> Table 1 shows that the HIV status of these children had little impact on the degree of malnutrition, as all children were selected on the basis of being hospitalized with PD and or chronically malnourished.

**Prevalence of *E. bieneusi* by HIV status.** Children with HIV infection were more likely to have *E. bieneusi* in their stool than HIV negative children. Of the 91 HIV-positive children, 70 (76.9%) were excreting *E. bieneusi*, compared with only 10 (6.6%) of the 152 without HIV (odds ratio = 47.33; 95% CI = 19.88 to 115.97; *P* < 0.0001). Children with CD4 lymphocyte counts below 25% were more likely to excrete *E. bieneusi* than those with higher percentage (24% versus 9%; odds ratio = 7.42; 95% CI = 3.77 to 14.69; *P* = 0.002). Overall, children with *E. bieneusi* were more likely to be older and have a lower percentage of CD4 cells than children free of the infection. Among the clinical features, only HIV status was significantly associated with *E. bieneusi* (Table 2).

**Prevalence of *Cryptosporidium* by HIV status.** Overall, 76

TABLE 2  
Clinical features of children by *Enterocytozoon bieneusi* status

Clinical feature	Adjusted odds ratio (95% CI) <sup>*</sup>	P value
Severe dehydration	2.51 (0.19–33.68)	0.486
Lymphadenopathy	0.67 (0.12–3.67)	0.641
CD4 cell %		
> 25	1.00	
> 15 $\leq$ 25	0.76 (0.07–1.85)	0.818
$\leq$ 15	0.18 (0.12–1.85)	0.148

<sup>\*</sup> Adjusted for HIV status.

(31.3%) of the 243 children with PD were excreting *Cryptosporidium* oocysts in the stool. Children with HIV were more likely to have *Cryptosporidium* oocysts than children without. Of the 91 children with HIV, 67 (73.6%) had *Cryptosporidium*, compared with only 9 (5.9%) of the 152 children without (odds ratio = 44.36; 95% CI = 18.39 to 110.40;  $P < 0.0001$ ). Children with low CD4 cell percentages (< 25%) were more likely to have *Cryptosporidium* than those with higher percentages (odds ratio = 6.45; 95% CI = 3.28 to 12.76;  $P < 0.0001$ ). Clinical features of children excreting *Cryptosporidium* oocysts are shown in Table 3. Only HIV infection was independently associated with *Cryptosporidium*.

Genotypic analysis of the *Cryptosporidium* DNA on 76 children showed that 56 (73.7%) were the anthroponotic *C. hominis* (or Type 1), 14 (18.4%) were the zoonotic *C. parvum* (or Type 2), 3 (3.9%) were a mixture of both, and 3 (3.9%) were *C. meleagridis*, the avian species. Although the numbers were too small for a firm conclusion, *C. parvum* was significantly more prevalent among children with HIV than *C. hominis* (odds ratio = 0.167; 95% CI = 0.036 to 0.771;  $P = 0.014$ ), and was more likely to occur together with *E. bienewsi* ( $P = 0.03$ ; Fisher exact test), indicating a possible common source of infection.

**Co-infection with *E. bienewsi* and *Cryptosporidium*.** Of the 243 children, 67 (27.8%) were excreting both *E. bienewsi* and *Cryptosporidium* in their stool. This means that 67 (83.8%) of the 80 children with *E. bienewsi* had *Cryptosporidium*; and 67 (88.2%) of the 76 children with *Cryptosporidium* had *E. bienewsi*. Factors associated with coinfection are shown in Table 4. However only infection with HIV was independently associated with coinfection.

One or both microorganisms were present in the stool of 89 (36.6%) of the 243 PD children recruited for this study, and in 74 (81.3%) of the 91 children with HIV, compared with 15 of 152 (9.9%) children without.

**Severe dehydration.** Although only 18 (7.4%) of the 243 children with PD had severe dehydration, the case fatality rate was high (35.7%), compared with only 12.8% among children who were not severely dehydrated. Factors associated with severe dehydration included *E. bienewsi*, oral thrush and others as shown in Table 5. After regression analysis, only oral thrush was independently associated with severe dehydration.

DISCUSSION

There have been several studies on cryptosporidiosis from Africa.<sup>8-16</sup> These early studies focused either on adults with AIDS, or on children but with no specific reference to their HIV/AIDS and immune status, or to the nature of the para-

TABLE 3  
Clinical features of children by *Cryptosporidium* status

Feature	Adjusted odds ratio (95% CI)*	P value
Dehydration	0.63 (0.15-2.67)	0.526
Lymphadenopathy	0.61 (0.11-3.32)	0.565
CD4 cell %		
> 25	1.00	
> 15 ≤ 25	1.13 (0.11-11.81)	0.919
≤ 15	6.43 (0.64-64.74)	0.114

\* Adjusted for HIV status.

TABLE 4  
Factors associated with coinfection with *E. bienewsi* and *Cryptosporidium* among children with persistent diarrhea

Feature	Adjusted odds ratio (95% CI)*	P value†
Dehydration	0.77 (0.17)	0.735
Lymphadenopathy	1.19 (0.22-6.43)	0.838
CD4 cell %		
> 25	1.00	
> 15 ≤ 25	0.99 (0.09-11.19)	0.992
≤ 15	5.97 (0.60-59-18)	0.127

\* Adjusted for HIV status; CI = confidence level.  
† P value is significant at the < 0.05 level.

sites they harbored. Recently used sophisticated molecular epidemiologic techniques have increased the sensitivity of detection, as well as allowing for a simultaneous genetic characterization of the pathogens isolates obtained from patients. There are no publications from Africa, or from anywhere else, addressing the epidemiology, natural history, and the clinical significance of microsporidiosis in children with HIV/AIDS. There are a few that report *E. bienewsi* infections in African adults with HIV/AIDS.<sup>1,2,21</sup> To an extent, this publication fills some of these gaps.

In this study we determined the prevalence of both *E. bienewsi* and *Cryptosporidium* among children with PD (lasting > 14 days), in whom their HIV and immune status (CD4 cell count) were determined. Whereas similar information had been previously reported in a few studies of adult patients with HIV,<sup>2,3</sup> our series appears to be the largest record of African children infected with HIV and PD who were excreting *E. bienewsi* and *Cryptosporidium*. That *E. bienewsi* and *Cryptosporidium* were found in children with PD was not surprising and is consistent with previous observations in this<sup>22,23</sup> and in other populations.<sup>1,11,17</sup> What was astonishing is the very high percentage of HIV-infected children with PD who were excreting either *Cryptosporidium* (73.6%), *E. bienewsi* (76.9%), both (69.2%), or one or both (81.3%). In contrast, only 8.5% of 1,779 children without diarrhea, sampled at Mulago Hospital, were excreting *Cryptosporidium*, regardless of HIV or immune status.<sup>22</sup> This is higher than in any other recorded HIV population, including adults. This may be, as stated earlier, due to the sensitivity of the genetic methods used in these studies, rather than an exceptionally high prevalence in these children. These observations indicate that these two pathogens alone may in fact be responsible for the great majority of PD in African children with HIV/AIDS. As in our previous study on *E. bienewsi*,<sup>23</sup> there was no significant association between infection in this subpopulation

TABLE 5  
Factors associated with severe dehydration among children with persistent diarrhea

Factor	Adjusted odds ratio (95% CI)*	P value
<i>E. bienewsi</i>	1.60 (0.06-45.98)	0.784
Oral thrush	9.62 (2.36-39.16)	0.002
Fever	3.54 (0.73-17.27)	0.117
CD4 cell %		
> 25	1.00	
> 15 ≤ 25	0.29 (0.06-1.42)	0.128
≤ 15	0.32 (0.06-1.78)	0.192

\* Adjusted for HIV status.



and malnutrition even after controlling for HIV status and CD4 counts/percentages.

Children with *E. bieneusi* or *Cryptosporidium* had more advanced HIV disease, as shown by lower CD4 cell counts and percentages, further confirming the view that these pathogens seem to be markers of advanced AIDS.<sup>25</sup> The outcome was unfavorable for those with severe dehydration and low CD4 cell counts. There are limited observations on coinfection from sub Saharan Africa. In the current study the risk factors for the coinfection were infection with HIV, low CD4 cell percentages, and generalized lymphadenopathy. This implies that HIV-positive children who have PD need to be tested for both of these opportunistic enteric infections.

Consistent with our earlier observations,<sup>22</sup> the ratio between the prevalence of *C. hominis*, the anthroponotic species (74%), and *C. parvum*, the zoonotic species (18%), was maintained, indicating that this ratio is a stable phenomenon. This is significant and indicates that infection is acquired directly from human contact, or indirectly through drinking water contaminated with human effluent. This is because *C. hominis*, unlike *C. parvum*, predominantly perpetuates in humans. The limited data that *C. parvum* occurred at higher frequency in children with HIV may have clinical and epidemiologic implications. One plausible explanation for the higher frequency of *C. parvum* infections in children with HIV is that these children are probably exposed to cryptosporidiosis multiple times and once they are exposed to *C. parvum*, it predominates thereafter. This is based on our previous work in which *C. parvum* was shown to be predominant over *C. hominis* in an infected host.<sup>26</sup> Observations that *C. parvum* was also found more frequently with *E. bieneusi* than was *C. hominis* may point to a possible common source of infection. Both of these observations, however, need to be further investigated with a larger number of children. It is important to identify the major sources of these pathogens, through community based epidemiologic studies, with a view to formulate appropriate control measures.

Although nitazoxanide and fumagillin are drugs that have been used to treat cryptosporidiosis and microsporidiosis, respectively, neither is effective in eradicating these chronic infections in HIV-infected individuals and consequently are not commonly used for such patients. In addition, nitazoxanide is not yet FDA-approved for use in immunocompromised individuals,<sup>27</sup> and fumagillin is considered toxic for children.<sup>28</sup> As experience with ART in African children with HIV is limited, the impact of this treatment on cryptosporidiosis and microsporidiosis in these children who are often malnourished is also unknown. Monitoring the impact of ART on these pathogens will hopefully be the next step.

Although there are several rapid staining methods used for the diagnosis of microsporidia spores in stool specimens,<sup>28</sup> they are nonspecific for *E. bieneusi*, the microsporidia we had targeted for these studies, as distinct from others including *Encephalitozoon intestinalis*. In contrast, the molecular genetic tools we have used, while laborious and not commonly used in routine clinical diagnosis, provided superior specificity and sensitivity. The PCR technique that has been in practice in this laboratory for a decade has, in addition to greater specificity and sensitivity, the major advantage of detecting both pathogens simultaneously, including genotyping in the case of cryptosporidiosis and sequence analysis of *E. bieneusi*.

**Conclusion.** *E. bieneusi* and *Cryptosporidium* are highly

prevalent in Ugandan children, especially those immunocompromised by HIV/AIDS. Coinfection with both organisms occurs in 81% of those with HIV/AIDS. There is a need to introduce ART in these children to control HIV and indirectly PD that is associated with these two enteric infections.

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