Multiplex Real-time PCR Detection of Intestinal Protozoa in HIV-infected Children in Malawi: Enterocytozoon Bieneusi Is Common and Associated With Gastrointestinal Complaints and May Delay BMI (Nutritional Status) Recovery

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Citation

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Enterocytozoon Bieneusi Is Common and Associated With Gastrointestinal Complaints and May Delay BMI (Nutritional Status) Recovery

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Background: Intestinal protozoa are common opportunistic infections in HIV patients. Longitudinal studies on either the clinical relevance or the effect of immune reconstitution by antiretroviral therapy on intestinal protozoan infections in children are lacking however. This study investigates prevalence and clinical relevance of intestinal protozoa in HIV-infected Malawian children before and during their first year of antiretroviral treatment (ART).

Methods: Stool samples collected at enrolment and during follow-up were tested for nonopportunistic (*Giardia lamblia, Dientamoeba fragilis, Entamoeba histolytica*) and opportunistic protozoa (*Enterocytozoon bieneusi, Encephalitozoon* spp., *Cryptosporidium* spp. and *Cystoisospora belli*) using multiplex real-time polymerase chain reaction. Associations between infections and clinical symptoms were evaluated using univariate methods.
Results: Nonopportunistic and opportunistic protozoa were detected in 40% (14/35) and 46% (16/35) of children at baseline, respectively. *E. bieneusi* was the most prevalent protozoa (37%, 13/35) and associated with gastrointestinal complaints (43% in positive (10/13) versus 18% (4/22) in *E. bieneusi*-negative children, $P = 0.001$. Body mass index recovery during 12 months of ART was more commonly delayed in *E. bieneusi*-positive children (+0.29 +standard deviation 0.83) than *E. bieneusi*-negative children (+1.03 +standard deviation 1.25; $P = 0.05$). *E. bieneusi* was not detected after 12 months of ART.
Conclusions: *E. bieneusi* was the most prevalent opportunistic intestinal protozoa, present in over a third of study participants before initiation of ART. Although all children cleared *E. bieneusi* after 12 months of ART, *E. bieneusi* was associated with gastrointestinal complaints and may delay body mass index recovery. Trials to assess effect of treatment of *E. bieneusi* on nutritional status should be considered in HIV-infected African children.

Key Words: intestinal protozoa, microsporidiosis, *Giardia lamblia, Dientamoeba fragilis, Entamoeba histolytica, Enterocytozoon bieneusi, Encephalitozoon* spp., cryptosporidium, HIV, antiretroviral treatment

Intestinal protozoal infections, caused either by opportunistic or nonopportunistic species, can complicate HIV infection in adults and children. Protozoal infections can cause chronic diarrhea and thereby malabsorption leading to malnutrition and dehydration, contributing to the high rates of morbidity and mortality observed in resource-limited settings.[1–3] Prevalence data on protozoal infections in patients with chronic diarrhea range widely between 1% and 75%, which can be explained by differences in demographics, seasonal variance and diagnostic methods.[4,5] Prevalence rates are higher in those studies employing modern, more sensitive, polymerase chain reaction (PCR) techniques.[6,7] However, PCR techniques are not commonly available in resource-limited settings and therefore reliable data from these areas are scarce.

Treatment of HIV infection has greatly improved over the past decade because of the massive scale up of antiretroviral treatment (ART) availability. In general, ART reduces HIV viral load, permits immune reconstitution and reduces the risk of new opportunistic infections.[8] Reduction of the prevalence of intestinal protozoa in HIV-infected adults after introduction of ART has been documented both in industrialized nations and resource-limited

settings.[9–11] However, prospective data on children receiving ART are lacking. Longitudinal data are needed to clarify which opportunistic infection may resolve through immune reconstitution because of ART alone and which infections might require more targeted therapy.

Therefore, this prospective cohort study was conducted to document the prevalence of opportunistic and nonopportunistic intestinal protozoa in HIV-infected children in Malawi, during the first year of first-line ART, using multiplex real-time PCR techniques. Additionally, the study investigated the dynamics and clinical aspects of the most common infections to evaluate their contribution to clinical symptoms and morbidity in Malawian children.

MATERIALS AND METHODS

Study Population

The study cohort included ART-naïve HIV-infected children initiating ART at the Queen Elizabeth Central Hospital, Blantyre, Malawi. Children 18 months to 18 years of age were enrolled before initiating ART, if they met ART initiation criteria outlined in the Malawi National ART program guidelines (2010–2012).[12,13] Local guidelines, applicable at the time of the study have been described in detail elsewhere.[14] In short, ART was initiated in children based on World Health Organization (WHO) staging and immune status. Clinical criteria included: ≥18 months of age with WHO clinical stage 3 and severe immune suppression was based on the WHO guideline at the time of the study: for children <59 months of age by CD4% <25% and/or CD4 <750 cells/mm and children >59 months of age CD4 <350 cells/mm.[12]

Data Collection

Following recruitment and informed consent, a standardized history was collected, including socioeconomic data and gastrointestinal symptoms. A physical examination was performed including collection of anthropometry. Children were monitored via data collection evaluating symptoms and anthropometry throughout the first 12 months of ART. Blood and stool samples were collected at 0, 6 and 12 months. This intestinal protozoa study was nested within a larger ART follow-up cohort study. Stool samples were stored and determined at a later point in time. All physicians were unaware of the PCR results.

Laboratory

HIV infection was confirmed using 2 HIV antibody tests (Abbott Determine HIV-1/2 Test and Uni-Gold HIV test). Full blood count (Beckman Coulter HMX, Beckman coulter, CA) and CD4 count (flow cytometry, Becton Dickinson, CA) were analyzed at the MLW Clinical Research Programme laboratory. Aliquots were stored for batch analysis of HIV-1 viral load (Roche Amplicor; Roche, Basel, Switzerland). Stool samples were stored at −20°C within 24 hours after sampling and subsequently shipped to Leiden University Medical Center, Leiden, The Netherlands. DNA isolation, amplification and detection were performed at Leiden University Medical Center as described elsewhere with some modifications, in particular the combinations of targets.[15–17] One multiplex real-time PCR was used for the detection of DNA of the nonopportunistic protozoa *Giardia lamblia*, *Dientamoeba fragilis* and *Entamoeba histolytica*[15] and another multiplex real-time PCR targeted opportunistic protozoa including microsporidia *Enterocytozoon bieneusi and Encephalitozoon* spp. (ie, *Encephalitozoon intestinalis*)[18] with *Cryptosporidium* spp.[16] and *Cystoisospora belli*.[17] Appropriate positive and negative controls were included in each assay. The PCR output consisted of a cycle threshold (Ct-) value representing the amplification cycle in which the level of fluorescent signal exceeded the background fluorescence. Intensity of infection was categorized for each target into low, moderate and high DNA levels based on Ct value, respectively: higher than 35, between 35 and 30 and lower than 30, while negative PCR results were recoded as Ct = 50.[19]

Definitions

Gastro-intestinal symtoms, including abdominal pain, diarea and vomiting, were assist at each planned visit. Anemia was classified using age and gender-specific hemoglobin (Hb) cutoffs: children 18–59 months of age, Hb <11.0 g/dL, children 5.0–11.9 age of years <11.5 g/dL, boys 12–15 years of age Hb <12.0 g/dL, girls >12 years of age; Hb 12.0 g/dL; boys >15 years of age, Hb <13.0 g/dL.[20] Severe immune suppression was defined using the WHO definitions according to age[20]: 18–59 months of age, CD4% <10% or CD4 count <200 cells/mm³; >59 months of age, CD4 count <100 cells/mm³.[21] Anthropometric data, based on weight, length and age-related Z score, were calculated based on WHO Multicentre Growth Reference Study Group.[22,23] Primary outcome was BMI for age Z score (BMIZ) as this parameter was applicable to children of all ages while weight for age only applies to children <10 years and HAZ is a late marker for growth recovery (>6–12 months). Malnutrition was defined as a BMIZ less than −2 standard deviation (SD).[24]

Statistical Analysis

Data were double entered, cleaned and analyzed using SPSS version 19.0. The study was primarily powered to detect prevalence of protozoa at baseline and their change over time. Second, univariate analysis assessed risk factors for protozoal infection using χ^2 test or Fisher exact test for categorical variables and independent sample t test for continuous variables. All P values presented are 2 tailed and a significance level of <0.05 was used.

Ethical Considerations

The purpose of the study was explained to the guardians of each patient in Chichewa, and written informed consent was obtained before inclusion into the study from the parents or guardians and assent from the children, if applicable. The study protocol was approved by the Research Ethics Committee of the College of Medicine, University of Malawi.

RESULTS

A total of 35 children were included with a mean age of 7.9 (SD 4.2) years. Baseline characteristics are shown in Table (Supplemental Digital Content 1, http://links.lww.com/INF/C976). At baseline, severe immune suppression was prevalent among 30% (10/33). Prevalence of severe immune suppression did not differ significantly between the different age groups: 26% (5/19) in children <10 years of age, versus 36% (5/14) if >10 years of age; P = 0.56. Fifteen percentage (5/34) of children were malnourished (BMIZ less than −2 SD) at baseline, which dropped to 3% (1/33) after 12 months on ART. Gastrointestinal complaints occurred in 40% (14/35) of children during the first 6 months of follow-up, but these complaints were not associated to immunosuppression however (P = 0.411).

Stool samples were available for 35 children at baseline, 27 (77%) children at 6 months and 26 (74%) children at 12 months of follow-up.

Nonopportunistic Protozoa at Baseline

Nonopportunistic protozoa were detected in 40% (14/35) of the children at baseline (Table 1). *G. lamblia* was the most common protozoa and detected in 26% (9/35) of the samples. *D. fragilis*

TABLE 1. Multiplex Real-time PCR Results of Protozoal Infections in HIV-infected Children During the First Year of ART

	Baseline (n = 35)	6 mo (n = 27)	12 mo (n = 26)
Nonopportunistic protozoa			
Giardia lamblia			
Total positive	9 (26%)	4 (15%)*	3 (12%)†
High DNA level	2/9 (22%)	2/4 (50%)	1/3 (33%)
Moderate DNA level	5/9 (56%)	0/4 (0%)	1/3 (33%)
Low DNA level	2/9 (22%)	2/4 (50%)	1/3 (33%)
Dientamoeba fragilis			
Total positive	6 (17%)	6 (22%)‡	8 (31%)§
High DNA level	4/6 (67%)	2/6 (33%)	1/8 (13%)
Moderate DNA level	2/6 (33%)	2/6 (33%)	5/8 (63%)
Low DNA level	0/6 (0%)	2/6 (33%)	3/8 (34%)
Opportunistic protozoa			
Enterocytozoon bieneusi			
Total positive	13 (37%)	6 (22%)	0 (0%)
High DNA level	7/13 (54%)	3/6 (50%)	
Moderate DNA level	3/13 (23%)	2/6 (33%)	
Low DNA level	3/13 (23%)	1/6 (17%)	
Cryptosporidium spp.			
Total positive	4 (11%)	3 (11%)‖	0 (0%)
High DNA level	0/4 (0%)	1/3 (33%)	
Moderate DNA level	0/4 (0%)	1/3 (33%)	
Low DNA level	4/4 (100%)	1/3 (33%)	
Cystoisospora belli			
Total positive	0 (0%)	2**(7%)	0 (0%)
High DNA level		0/2 (0%)	
Moderate DNA level		1/2 (50%)	
Low DNA level		1/2 (50%)	

Entamoeba histolytica and *Encephalitozoon* spp. (ie, *Encephalitozoon intestinalis*) were not detected. Combined *E. bieneusi-Cryptosporidium* infection at baseline was seen in 3%; 1/35 and *E. bieneusi* combined with *G. lamblia* infection at baseline was seen among 11%; 4/35. Detected DNA level based on cycle threshold value: Total positive: cycle threshold < 50, low DNA level: cycle threshold 35–50, moderate DNA level: cycle threshold 30–35 and high DNA level: cycle threshold < 30.

*No new infections.
†2 new infections.
‡6 new infections.
§7 new infections.
¶4 new infections.
‖3 new infections.
**All new infections.

was prevalent in 17% (6/35) of children while *E. histolytica* was not detected. Three of 9 children (33%) with *G. lamblia* infection at baseline had gastrointestinal complaints. Gastrointestinal complaints among children with *G. lamblia* were not more common than among children without *G. lamblia* [42% (11/26), $P = 0.22$]. At baseline, BMIZ were −0.60 (SD 1.0) for *G. lamblia*-positive children versus a mean of −0.55 (SD 1.4) for negative children, $P = 0.92$. Also *D. fragilis* infections at baseline were not associated with gastrointestinal complaints or a significant difference in BMIZ scores. Respectively 50% (3/6) of the *D. fragilis*-positive children showed gastrointestinal complaints versus 38% (11/29) among *D. fragilis*-negative children, $P = 0.30$. BMIZ at baseline was +0.56 (SD 0.9) versus +0.55 (SD 1.4) in *D. fragilis*-positive and *D. fragilis*-negative children, respectively, $P = 0.99$.

Opportunistic Protozoa at Baseline

Opportunistic protozoa were detected in 46% (16/35) of children at baseline. *E. bieneusi* was the most common opportunistic protozoa, present in 37% of samples (13/35). Gastrointestinal complaints were more common among children with *E. bieneusi* at baseline than those without infection [43% (10/13) vs. 18% (4/22), $P = 0.001$; Table 2]. Children with and without *E. bieneusi* had a similar BMIZ at baseline; BMIZ −0.6 (SD 1.0) versus BMIZ −0.6

(SD 1.4), $P = 0.92$. The clinical relevance *E. bieneusi* infection is described in detail below. *Cryptosporidium* spp. was seen in 11% (4/35) of children, while *Encephalitozoon* spp. (ie, *E. intestinalis*) and *C. belli* were not detected at baseline. *Cryptosporidium* spp. infections at baseline were not associated with gastrointestinal complaints [42% (13/31) vs. 25% (1/4) for *Cryptosporidium* spp. positive and negative children, respectively; $P = 0.42$]. No difference in mean BMIZ at baseline was seen: −0.16 (SD 0.4) versus −0.57 (SD 1.3), $P = 0.56$ for *Cryptosporidium* spp. positive and negative children, respectively.

Effect of 12 Months ART on Intestinal Protozoa

The prevalence of nonopportunistic protozoa and opportunistic protozoa infections over time are described in Table 1. Nonopportunistic protozoa were present in 43% (15/35) of the children at baseline and 42% (11/26) after 12 months of ART. The prevalence of *G. lamblia* dropped from 26% (9/35) to 11% (3/26); however, new infections also occurred (Table 1). Prevalence of *D. fragilis* increased from 17% (6/35) toward 31% (8/26) after ART initiation. All opportunistic protozoa, including *E. bieneusi*, were cleared after 12 months of ART.

E. bieneusi

To identify children at risk for *E. bieneusi* and to assess the clinical importance of the infection, potential risk factors and symptoms were compared between children with and without *E. bieneusi* (Table 2). *E. bieneusi* was more common in children older than 10 years of age when compared with younger children [57% (8/14) vs. 24% (5/21), respectively; $P = 0.05$]. Children with *E. bieneusi*, when compared with children without, did not suffer from more severe immune suppression at baseline [38.5% (5/13) vs. 25.0% (5/15); $P = 0.46$]. However, children with and without *E. bieneusi* had a similar BMIZ at baseline; significant differences were seen after 12 months of ART. Children with an *E. bieneusi* infection showed a trend toward lower BMIZ when compared with those without; BMIZ −0.36 (SD 0.97) versus BMIZ +0.56 (SD 1.39), $P = 0.052$ (Fig. 1A). BMIZ recovery after 12 months was +0.29 (SD 0.83) versus +1.03 (SD 1.25; $P = 0.050$) for *E. bieneusi* infection versus noninfected children. Children with high levels of *E. bieneusi* DNA in their stool showed a nonsignificant trend toward a more delayed BMI recovery (Fig. 1B). BMIZ at 12 months of follow-up was −0.47 (SD 1.03), −0.20 (SD 0.97) versus +0.56 (SD 1.39) for high, low to moderate and negative baseline *E. bieneusi* DNA levels, respectively ($P = 0.25$).

DISCUSSION

This study presents important new PCR prevalence data evaluating nonopportunistic and opportunistic intestinal protozoa infections in HIV-infected African children receiving ART. *E. bieneusi*, an opportunistic infection, was the most prevalent intestinal protozoa. Despite being a rather unknown opportunistic infection among pediatric clinicians, *E. bieneusi* was not only common but also might be clinically relevant, as it was associated with both gastrointestinal complaints and possible reduced BMI recovery. Although all children cleared *E. bieneusi* by 12 months of ART and gastrointestinal complaints diminished, the BMI recovered poorly in those with an *E. bieneusi* infection.

Thirty-seven percentage of the ART naïve HIV-infected children were infected with *E. bieneusi* in this cohort. The reported prevalence of *E. bieneusi* in the literature shows wide ranges (0.8%–79%) of *E. bieneusi* prevalence. This may be explained by differences in diagnostic methods, demographics and study populations, including ages, presence of clinical symptoms, other underlying conditions and severity of the immune

TABLE 2. Factors Associated at Baseline and Over 12 Months Follow-up With *Encephalitozoon bieneusi* Infection at Baseline

	E. bieneusi-negative DNA Level	E. bieneusi-positive DNA Level	P	Odds Ratio (95% CI)
Sex: boys	9/22 (41%)	8/13 (62%)	0.31	0.4 (0.1–1.8)
Age, yr [mean (±SD)]	6.85 (±3.6)	9.8 (±4.5)	0.04	1.2 (1.0–1.5)
Symptoms				
Gastrointestinal symptoms*				
0–6 mo†	4/22 (18%)	10/13(43%)	0.001‡	15.0 (2.8–80.9)
Diarrhea				
0–6 mo†	1/22 (5%)	6/13 (46%)	0.003‡	18.0 (1.8–176.6)
Vomiting				
0–6 mo†	1/22 (5%)	7/13 (54%)	0.001‡	24.5 (2.5–240.3)
Immune status				
WHO				
I	10/22 (46%)	6/13 (46%)	0.85	-
II	5/22 (23%)	2/13 (15%)		
III	7/22 (32%)	5/13(39%)		
IV	0	0/39 (0%)		
Severe immune suppression§				
Baseline	5/20 (25%)	5/13 (39%)	0.41	1.9 (0.4–8.5)

*Gastrointestinal symptoms include abdominal pain or diarrhea or vomiting.
†Complaints during 0–6 mo follow-up.
‡*P* significant <0.05. Toilet use and water availability did not differ significant between children with and without *E. bieneusi infection* (data not shown).
§Severe immune suppression: age <59 mo: CD4% <10% or a CD4 count <200 cells/mm[3][20]; age >59 mo: CD4 count <100 cells/mm^{3}.[21]
CI indicates confidence interval; WHO, world Health Organization.

FIGURE 1. BMIZ of children with and without detectable *Enterocytozoon Bieneusi* DNA levels during 12 months of ART. BMIZ calculated based on WHO Multicentre Growth Reference Study Group.[20,21]. A: After 12 months follow-up, the BMIZ remained lower in children with *E. bieneusi* in comparison to those without *E. bieneusi* infection; BMIZ −0.36 versus BMIZ +0.56; *P* = 0.05. B: After 12 months follow-up, the BMIZ remained lower in children with *E. bieneusi* high DNA level in comparison to those with moderate to low and a negative *E. bieneusi* DNA level: BMIZ −0.47, −0.20 versus +0.56 for high, low to moderate and negative *E. bieneusi* DNA levels at baseline; *P* = 0.25. Detected DNA level based on Ct value: Low to moderate DNA level: Ct 30–50 and high DNA level: Ct < 30.
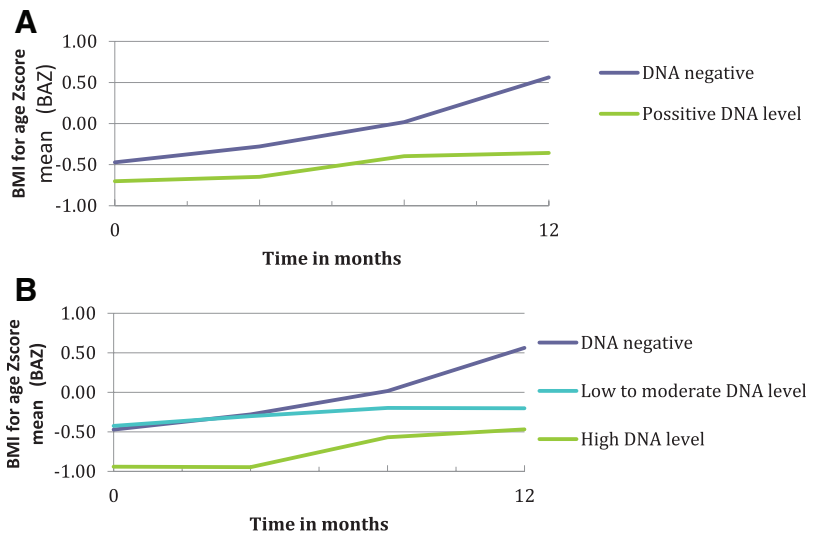
suppression.[25–27] Two other studies which used PCR-based assays in HIV-infected African children reported prevalence rates ranging from 20%—in a general population of HIV-infected children in South Africa to 79%—in children with HIV and diarrhea in Uganda of *E. bieneusi* infection.[5,27] In the current study, all children cleared *E. bieneusi* and other opportunistic protozoa following immune recovery after 12 months of ART. This is the first pediatric cohort study evaluating nonopportunistic and opportunistic intestinal protozoa infections including *E. bieneusi* during ART. However, the data collected in children are corroborating with data in adults showing clearance of the *E. bieneusi* infection after receiving several months of ART.[9,10] The finding that children clear opportunistic protozoa are especially reassuring as children are considered to be more vulnerable to *E. bieneusi* as it is more common in children than adults.[25]

Gastrointestinal complaints are commonly reported in microsporidial infections such as *E. bieneusi*.[28,29] In the current study, 43% of the children with a PCR-detected *E. bieneusi* infection described gastrointestinal complaints. The prevalence of gastrointestinal symptoms corroborates data in HIV-infected adults, where *E. bieneusi* infection has been associated with diarrhea and vomiting.[28,29] More importantly this cohort demonstrated that *E. bieneusi* is associated with a delayed BMI recovery, which persisted despite protozoal clearance and reduction of gastrointestinal complaints. Others, cross sectional studies have also reported an association between malnutrition, lower rates of weight gain and *E. bieneusi* in HIV infected patients.[28,30,31] Malnutrition and lower rates of weight gain are usually attributed to ongoing diarrhea and impaired absorption of micronutrients because of mucosal damage and malabsorption caused by direct replication of protozoa in the

epithelium of small intestine.[29] In this study, the gastrointestinal complaints resolved within 6 months and immune status improved during 12 months of ART, while a poor nutritional state, signified by delayed BMI recovery, persisted. Second, there was no association between potential confounders such as immune suppression at baseline (Table 2), neither tuberculosis infection at enrolment nor ART failure at 12 months and BMI recovery in this population (data not shown). Further studies are needed to assess if the effect on BMI is secondary to a direct effect of *E. bieneusi* on the mucosa or if more factors are involved in the delayed BMI recovery.

Like other studies, a large part of patients with an *E. bieneusi* infection detected by PCR are asymptomatic.[5,25] However, other studies have not assessed if the lower protozoal load may explain why some do not have symptoms.[5,25] Low detectable DNA levels may, for instance, reflect spore shedding rather than actual infection.[6,31–33] *E. bieneusi* spores are detected in stool specimens by microscopy for 9–33 days, while stool specimens evaluated by PCR are positive for 3–40 days longer.[25] PCR results should therefore be interpreted with some care, especially if low DNA levels are detected. Despite this, we did identify a trend toward a poorer BMI recovery among children with high *E. bieneusi* DNA levels in this relatively small study.

None of the children received treatment after their PCR results in this study. Given our finding of a significant association between *E. bieneusi* and a poor BMI recovery and clinical symptoms, the differences found were small and both may have multiple causes. Therefore, the effect of therapy for *E. bieneusi* on these outcomes should be tested in a placebo-controlled trial. Effective treatment for *E. bieneusi* is complex, however. Albendazol has shown conflicting results in the treatment of different *E. bieneusi* subtype infections.[34,35] Fumagillin, a newer agent, was shown to be effective in adults and was approved in 2005, but severe adverse effects limit its use and availability.[35,36] If these restrictions also apply to children is unclear, as paediatric data are very limited. However data available show good effectiveness and no side effects.[36] TPN 470, the fumagillin analogue, may be a potentially safer alternative though it again lacks pediatric testing.[2,34,35] Given the serious reduced BMI recovery in these HIV-infected children, phase 2 and 3 trials should be considered.

Besides *E. bieneusi, G. lamblia* was a common nonopportunistic pathogen. Prevalence of *G. lamblia* worldwide can differ enormously due to diagnostic test, population, demographics, season and ages.[37,38] Our reported PCR-detected prevalence of 26% is similar to a previously reported prevalence of 30% among severely malnourished children in Blantyre, Malawi.[39] Immune status recovery has a questionable effect on infection while the prevalence of *G. lamblia* dropped from 26% toward 11% during 12 months of ART, new infections also occurred. *G. lamblia* infection can present with gastrointestinal complaints, but carriers may also be asymptomatic.[37,40] In this study, gastrointestinal complaints and malnutrition could not be linked to *G. lamblia* infection. As we detected mainly moderate and low DNA loads (78%), we may have identified a large proportion of asymptomatic carriers. As *G. lamblia* is not an opportunistic infection, it is unlikely that prevalence will be reduced by restored immunity due to ART.

Cryptosporidium spp. occurred in this study but infections resolved after immune recovery during ART and DNA levels were only high in 2 children. Earlier PCR-based studies in Malawi (Blantyre) among preschool children with diarrhea and an unknown HIV status showed a comparable prevalence of *Cryptosporidium* spp. of 5%–9%.[41] Cryptosporidiosis is commonly associated with symptoms of diarrhea; however, in this study, gastrointestinal complaints were not associated with infection.[42] This effect may be explained by the relatively low to moderate infection prevalence of all *Cryptosporidium* spp. infection at baseline as compared with

other pathogens. Considering the low prevalence and the lack of an effective treatment in children, routine testing will likely not benefit children in an outpatient setting.[43] Combined infections were not common among the presenting cohort. Clinical symptoms are therefore not related and influenced by coinfections.

This study had several limitations. First, this study used local guidelines to start ART in Malawi in 2010, which meant that the studied cohort was severely immune compromised in comparison to current cohorts of children starting ART. Current WHO guidelines suggest to start ART early in the course of HIV disease.[21] Current HIV-infected populations may therefore have lower protozoal prevalence rates. Given the high prevalence in our study and the fact that HIV diagnosis still is often delayed in African settings, we believe our findings are still important. Second, the number of children recruited is small and follow-up was stopped at 12 months; therefore, associations may have been missed or suggested that required a larger sample size or prolonged follow-up. Still this study is the first pediatric cohort from Africa using PCR techniques with 12 months of follow-up during ART. Third, the use of PCR may have overestimated the prevalence of pathogens over time as PCR also detects spores, which are not active pathogens.[6,31–33] However, we assessed the effect of DNA load and were able to show a trend toward a more delayed BMI recovery in children with a higher load. To confirm this effect, more research is needed to investigate the duration of diminished growth after 24 months and the eventual effect of (repeated presumptive) treatment on growth over time. Finally, we have not performed of *E. bieneusi* subtype analysis, this information could be of use as clinical presentation is influenced by genotype and therefore may vary in different geographic regions.[44,45]

This prospective cohort reports on the prevalence of intestinal protozoa in HIV-infected children in Malawi, during the first year of first-line ART using multiplex real-time PCR techniques. *E. bieneusi* is a very common pathogen at start of ART and clinically important as it was associated with gastrointestinal complains and may be associated with prolonged reduced growth, a predictor of poor prognosis. Future studies should focus on trials to assess treatment options for *E. bieneusi* to improve symptoms and poor nutrition status.

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