Unusual *Enterocytozoon bieneusi* Genotypes and *Cryptosporidium hominis* Subtypes in HIV-Infected Patients on Highly Active Antiretroviral Therapy

Frederick O. Akinbo, Christopher E. Okaka, Richard Omoregie, Haileeyesus Adamu, and Lihua Xiao*

Department of Medical Laboratory Science, University of Benin, Benin City, Nigeria; Department of Animal and Environmental Biology, University of Benin, Benin City, Nigeria; School of Medical Laboratory Sciences, University of Benin Teaching Hospital, Benin City, Nigeria; Division of Foodborne, Waterborne, and Environmental Diseases, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Diseases Control and Prevention, Atlanta, Georgia

Abstract. Human immunodeficiency virus (HIV)-infected persons are commonly infected with *Cryptosporidium* species and *Enterocytozoon bieneusi* in both developed and developing countries, particularly patients with CD4+ cell counts below 200 cells/μL; 285 HIV-infected patients on highly active antiretroviral therapy (HAART) were enrolled in this study, and both stool and blood specimens were collected from participants. The stool specimens were analyzed and typed for *E. bieneusi* and *Cryptosporidium* spp. by polymerase chain reaction (PCR) and DNA sequencing. CD4 count was analyzed using flow cytometry. *E. bieneusi* and *Cryptosporidium* were detected in 18 (6.3%) and 4 (1.4%) patients, respectively. The *E. bieneusi* detected mostly belonged to a new genotype group that, thus far, has only been found in a few humans: genotype Nig4 in 2 patients and two new genotypes related to Nig4 in 12 patients. The *Cryptosporidium* detected included *C. hominis* (two patients), *C. parvum* (one patient), and *C. felis* (one patient), with the two *C. hominis* infections belonging to an unusual subtype family. Additional studies are required to determine whether some *E. bieneusi* genotypes and *C. hominis* subtypes are more prevalent in HIV patients on HAART.

INTRODUCTION

Several opportunistic parasites have been implicated as major contributors to morbidity in immunocompromised persons, especially those persons living in developing countries. Human immunodeficiency virus (HIV)-infected persons have been reported to be commonly infected with *Cryptosporidium* species,[1–6] although the prevalence varies by location, age of the study population, stage of the HIV infection, and diagnostic methods used. Likewise, microsporidia have been identified as causes of opportunistic infections in both developed and developing countries, particularly in HIV-infected patients with CD4 cell count below 100 cells/μL.[7,8] Of the 15 species of microsporidia known to infect humans, *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* can cause gastrointestinal disease, with *E. bieneusi* being the more commonly identified species in HIV-infected persons.[9] In addition to causing human disease, *E. bieneusi* has also been frequently found in animals, especially mammals.[10,11] Microsporidia and *Cryptosporidium* develop in enterocytes, and they are excreted in feces and transmitted by the fecal–oral route, direct contact with infected persons or animals, or ingestion of contaminated food or water.[12,13]

Highly active antiretroviral therapy (HAART) has been reported to reduce the prevalence of microsporidiosis in HIV-infected patients in industrialized nations.[7,14] HAART promotes the reconstitution of the immune system of HIV-infected persons, thereby reducing the occurrence of opportunistic parasitic infections.[15–17] However, the effect of HAART on the prevalence of microsporidiosis in HIV-infected patients in developing countries remains unclear, where microsporidiosis is a major health problem because of the high prevalence of the disease in the general population and underfinanced healthcare systems.[18]

Molecular diagnostic methods, especially those methods with the capacity to genotype and subtype pathogens, have been used increasingly in the characterization of the transmission of *Cryptosporidium* spp. and *E. bieneusi* in HIV-infected persons.[5,19,20] In Nigeria, molecular epidemiologic studies of *E. bieneusi* and *Cryptosporidium* in HIV-infected persons have been conducted only in Benin City.[5,13] There is a paucity of information on epidemiology of *E. bieneusi* and *Cryptosporidium* in HIV-infected persons who are on HAART in developing countries. This study was, therefore, conducted to examine the prevalence and genetic characteristics of *E. bieneusi* and *Cryptosporidium* spp. among HIV-infected persons on HAART in Benin City, Nigeria.

MATERIALS AND METHODS

Study area. The study was carried out at the University of Benin Teaching Hospital, Benin City, Nigeria. It is located in the south–south geopolitical zone of Nigeria. It is within the low rainforest zone of Nigeria and has two seasons (dry and wet). It serves as a referral hospital for about 6–10 states in Nigeria. It is a center for the Institute of Human Virology, Nigeria and US President's Emergency Plan for AIDS Relief (PEPFAR).

Study population. Two hundred eighty-five HIV-infected adults on HAART attending the hospital were enrolled in this study. Individuals on antiparasitic agents and individuals with AIDS-defining illnesses were excluded from this study. A structured questionnaire was used to collect demographic data and clinical signs and symptoms from each participant. Informed consent was obtained from all study participants before specimen collection. Ethical clearance to carry out the study was sought from the Ethical Committee of the University of Benin Teaching Hospital, Benin City, Nigeria, with approval number ADM/E.22A/Vol.VII/29.

Specimen collection. Stool and blood specimens were collected from participants. The blood specimens were placed in ethylene diamine tetra acetic acid (EDTA) containers, whereas the stool specimens were preserved in 2.5% potassium

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*Address correspondence to Lihua Xiao, Division of Foodborne, Waterborne, and Environmental Diseases, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Diseases Control and Prevention, Atlanta, GA. E-mail: lxiao@cdc.gov

dichromate solution at 4°C. Aliquots of the stool specimens were shipped to the laboratory at the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, for the detection and genotyping of *E. bieneusi* and *Cryptosporidium* spp.

Analysis of blood specimens. CD4+ T lymphocyte cell count was analyzed using flow cytometry (Partec, GmbH, Münster, Germany) and the manufacturer-recommended procedures. Hemoglobin concentration was analyzed using the Sysmex KX 21 (Sysmex Corporation, Chuo-ku, Kobe, Japan). Hemoglobin levels < 12 g/dL for females and < 13 g/dL for males were used to define anemia.

Detection and typing of *E. bieneusi* and *Cryptosporidium*. The stool specimens were washed in distilled water by centrifugation. DNA was extracted using the FastDNA SPIN kit for soil (BIO 101, Carlsbad, CA). For the detection of *E. bieneusi*, each DNA sample was analyzed in duplicate by a nested polymerase chain reaction (PCR) targeting an approximately 392-bp region of the partial 18S rRNA gene, the entire internal transcribed spacer (ITS), and the partial 5.8S rRNA gene.[21] For the detection and differentiation of *Cryptosporidium* spp., the DNA preparations were analyzed by an 18S rRNA gene-based PCR restriction fragment-length polymorphism (RFLP) technique.[22] The *C. hominis* and *C. parvum* recovered were subtyped by PCR sequence analysis of the 60 kDa glycoprotein (gp60) gene.[23] The PCR products were stained in GelRed™ (Biotium Inc., Hayward, CA), analyzed by agarose gel electrophoresis, and visualized under ultraviolet illumination.

Nucleotide sequence analysis. Positive ITS and gp60 PCR products were purified and sequenced in both directions using the Big Dye Terminator Cycle Sequencing Kit on an ABI 3130 automated sequencer (Applied Biosystem, Foster City, CA). The accuracy of nucleotide sequences was confirmed by sequencing of two independent PCR products. *E. bieneusi* genotypes and *C. hominis* or *C. parvum* subtypes were determined by aligning the ITS or gp60 sequences obtained with reference sequences using the program ClustalX (http://www.clustal.org/). The established nomenclature systems were used in naming *E. bieneusi* genotypes and *C. hominis* and *C. parvum* subtypes.[24,25] The genetic relationship among *E. bieneusi* genotypes was assessed by a neighbor-joining analysis of the aligned sequences using the program Treecon (http://bioinformatics.psb.ugent.be/software/details/3) and Kimura two-parameter distances. Bootstrap analysis was used to assess the reliability of grouping using 1,000 pseudo-replicates. Unique nucleotide sequences generated from the study were submitted to GenBank under accession numbers JX524489–JX524505.

Statistical analysis. The frequency data obtained in this study were compared using the χ^2 test, and odd ratios (ORs) were calculated for each potential risk factor using the software INSTAT (GraphPad Software Inc., La Jolla, CA).

RESULTS

Of 285 HIV-infected patients on HAART enrolled in this study, *E. bieneusi* and *Cryptosporidium* were detected in 18 (6.3%) and 4 (1.4%) patients, respectively. The infection rate of *E. bieneusi* infection was significantly ($P = 0.006$) higher in males (13.1%) than females (3.5%), and the male sex was a risk factor for *E. bieneusi* infection (OR = 4.176; 95% confidence interval [95% CI] = 1.559, 11.187). No sex difference was seen in the occurrence of cryptosporidiosis (OR = 1.26; 95% CI = 0.13, 12.27 for the female sex). CD4+ cell count less than 200 cells/μL was significantly associated with *E. bieneusi* infection (OR = 19.09; 95% CI = 6.38, 57.12) and cryptosporidiosis (OR = 52.16; 95% CI = 2.76, 987.56; $P < 0.0001$). Diarrhea was significantly associated with *E. bieneusi* infection (OR = 3.563; 95% CI = 1.295, 9.797; $P = 0.019$). Anemia was not associated with *E. bieneusi* infection (OR = 1.225; 95% CI = 0.471, 3.184; $P = 0.863$). Both diarrhea ($P = 0.307$) and anemia ($P = 0.09$) were not significantly associated with the occurrence of cryptosporidiosis (Table 1).

TABLE 1
Risk factors involved in the occurrence of *E. bieneusi* infection in HIV patients on HAART

| Risk factor	No. tested	No. with infection	Infection rates (%)	OR	95% CI	P value
E. bieneusi						
Male	84	11	13.1	4.78	1.56, 11.19	0.005*
Female	201	7	3.5	0.24	0.09, 0.64	
Cryptosporidium						
Male	84	1	1.2	0.80	0.08, 7.78	0.843
Female	201	3	1.5	1.26	0.13, 12.27	
E. bieneusi						
CD4+ count < 200 cells/μL	45	13	28.9	19.09	6.38, 57.12	< 0.0001*
CD4+ count ≥ 200 cells/μL	240	5	2.1	0.05	0.02, 0.17	
Cryptosporidium						
CD4+ count < 200 cells/μL	45	4	8.9	52.16	2.755, 987.56	< 0.0001*
CD4+ count ≥ 200 cells/μL	240	0	0	0.02	0.01, 0.36	
E. bieneusi						
Diarrhea	108	12	11.1	3.56	1.30, 9.80	0.019*
No diarrhea	177	6	3.4	0.28	0.10, 0.78	
Cryptosporidium						
Diarrhea	108	3	2.8	5.03	0.52, 49.00	0.307
No diarrhea	177	1	0.6	0.20	0.02, 1.94	
E. bieneusi						
Anemia	129	9	7.0	1.23	0.47, 3.18	0.863
No anemia	156	9	5.8	0.82	0.31, 2.12	
Cryptosporidium						
Anemia	129	4	3.1	11.23	0.60, 210.58	0.089
No anemia	156	0	0	0.09	0.01, 1.67	

*$P < 0.05$.
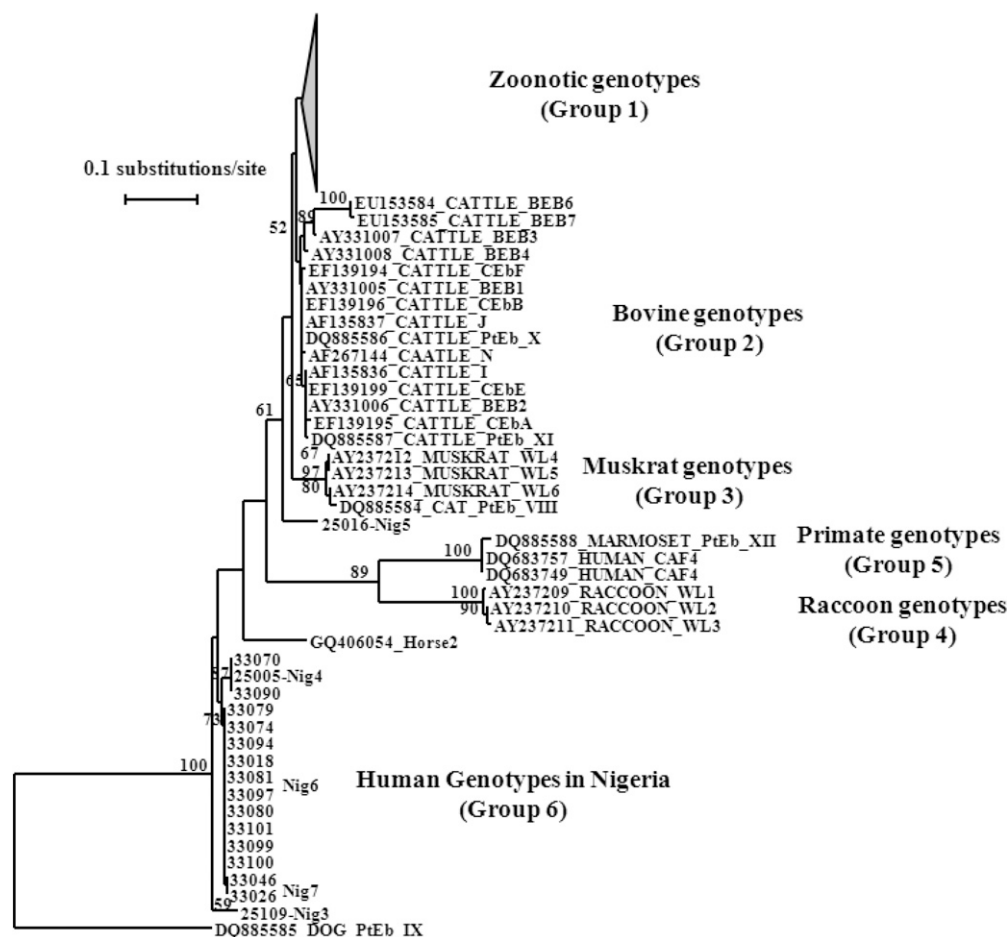
FIGURE 1.   Genetic uniqueness of *E. bieneusi* genotypes in HIV-infected Nigerian patients as indicated by a neighbor-joining analysis of ITS sequences based on genetic distances calculated by the Kimura two-parameter model. The genotype group names are based on the works by Breton and others[19] and Sulaiman and others.[21] Each sequence from GenBank is identified by the accession number, host origin, and original genotype designation. Except for one patient with Type IV, most patients in the study were infected with members (Nig4, Nig6, and Nig7) of a new genotype group (Group 6) unique to HIV patients in Nigeria.

The *E. bieneusi* detected belonged to four genotypes. Two established genotypes, Type IV and Nig4, were detected in one and two patients, respectively. Two new genotypes related to Nig4, named Nig6 and Nig7, were detected in 10 and 2 patients, respectively (Figure 1). The new genotypes had four and five nucleotide substitutions compared with Nig4, respectively. Three specimens had concurrent occurrence

of mixed *E. bieneusi* genotypes, which was reflected by the production of noisy sequences in DNA sequencing (Table 2).

In contrast, three *Cryptosporidium* species, including *C. hominis* (2), *C. parvum* (1), and *C. felis* (1), were detected (Table 2). The *C. parvum* belonged to the subtype IIcA5G3a, whereas the two *C. hominis* isolates produced gp60 sequences of a new subtype family related to the Id subtype family, with eight nucleotide substitutions in the region after the trinucleotide repeats.

TABLE 2

E. bieneusi and *Cryptosporidium* genotypes detected in 285 HIV patients on HAART

Genotype	Occurrence	Percent
E. bieneusi		
Type IV	1	5.6
Nig4	2	11.1
Nig6	10	55.6
Nig7	2	11.1
Mixed genotypes	3	16.7
Total	18	
Cryptosporidium		
C. hominis	2	50
C. parvum	1	25
C. felis	1	25
Total	4	

DISCUSSION

The infection rates of 6.3% and 1.4% of microsporidiosis and cryptosporidiosis, respectively, in this study were lower than the 16.6% infection rate of microsporidiosis and the 9% infection rate of cryptosporidiosis previously reported in HAART-naïve HIV patients in the same locality.[4,5,12] It has been reported that HAART reduces the prevalence of opportunistic infections, including microsporidiosis and cryptosporidiosis, by reconstitution of immunity.[26,27] In several previous studies,[8,27] no microsporidiosis was detected in HIV-positive patients after several years of HAART. In contrast, cryptosporidiosis in HAART patients has been reported in some studies

in developing countries.[28,29] The difference in microsporidiosis occurrence between these studies and our study could be because of differences in the duration of HAART; in our study, patients had been on HAART for only 3–6 months.

The reason for male sex being a risk factor for *E. bieneusi* infection among HIV patients on HAART is unclear, because the male sex was not reported to be associated with *E. bieneusi* infection in HAART-naïve HIV patients.[13] In addition, male sex was not a risk factor for cryptosporidiosis occurrence in this study and previous studies.[30]

The finding that CD4+ cell counts < 200 cells/µL were significantly associated with *E. bieneusi* and *Cryptosporidium* infections is in agreement with many studies in HAART-naïve HIV patients.[1,4,5,13] In a Brazilian study of patients on HAART, the occurrence of cryptosporidiosis was also mostly in patients with CD4+ cell counts below 200 cells/µL.[31] Although HAART improves immunity, HIV patients on HAART with CD4+ cell count less than 200 cell/µL are still prone to opportunistic infections. Our patients had only been on HAART for 6 months; longer duration of HAART probably would lead to full immune reconstitution and clearance of *Cryptosporidium* and *E. bieneusi* infections.

Diarrhea was significantly associated with the occurrence of microsporidiosis ($P = 0.0188$). This observation is consistent with the finding of Akinbo and others[13] in HAART-naive HIV patients. The small number of cryptosporidiosis cases has prevented us from assessing the association of cryptosporidiosis and the occurrence of diarrhea, but such an association was seen in previous reports in Nigeria.[2,4,5] Anemia had no significant association with *E. bieneusi* infection. The cause of anemia in HIV patients is multifactorial and includes opportunistic infections, neoplasm, dietary deficiencies, blood loss, medications, and antibodies to antiretroviral agents.[32] It would, thus, seem that *E. bieneusi* is not among the agents causing anemia in HIV patients. Likewise, the small number of cryptosporidiosis cases has prevented us from assessing its association with anemia. However, *Cryptosporidium* infection was shown previously associated with the occurrence of anemia in HAART-naïve HIV patients.[33]

In this study, the distribution of *E. bieneusi* genotypes seems to be different from that identified previously in HAART-naïve HIV patients and children in Nigeria. Compared with the occurrence of common genotypes such as A, D, Type IV, and WL7 in Group 1 in previous studies,[13,34,35] the *E. bieneusi* genotypes detected in this study mostly belonged to Nig4 and two new genotypes related to Nig4. Because this group of genotypes is very divergent from other known genotype groups and seems to be specific to humans, we have named the new genotype group as Group 6. Likewise, the *C. hominis* Id-like subtype family seen in two patients in this study has never been reported in humans in previous studies in Nigeria.[5,6,35,36] Additional studies are required to determine whether some *E. bieneusi* genotypes and *C. hominis* subtypes are more common in HIV patients on HAART.

Authors' addresses: Frederick O. Akinbo, Department of Medical Laboratory Science, School of Basic Sciences, University of Benin, Benin City, Nigeria, E-mail: fgbengang@yahoo.com. Christopher E. Okaka, Department of Animal and Environmental Biology, Faculty of Life Sciences, University of Benin, Benin City, Nigeria, E-mail: drceokaka@yahoo.com. Richard Omoregie, School of Medical Laboratory Sciences, University of Benin Teaching Hospital, Benin City, Nigeria, E-mail: richyomos@yahoo.com. Haileeyesus Adamu and Lihua Xiao, Division of Foodborne, Waterborne, and Environmental Diseases, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Diseases Control and Prevention, Atlanta, GA, E-mails: haile27@ymail.com and lxiao@cdc.gov.

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