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# Cryptosporidiosis in HIV/AIDS Patients in Kenya: Clinical Features, Epidemiology, Molecular Characterization and Antibody Responses

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*Abstract.* We investigated the epidemiological and clinical features of cryptosporidiosis, the molecular characteristics of infecting species and serum antibody responses to three *Cryptosporidium*-specific antigens in human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) patients in Kenya. *Cryptosporidium* was the most prevalent enteric pathogen and was identified in 56 of 164 (34%) of HIV/AIDS patients, including 25 of 70 (36%) with diarrhea and 31 of 94 (33%) without diarrhea. Diarrhea in patients exclusively infected with *Cryptosporidium* was significantly associated with the number of children per household, contact with animals, and water treatment. *Cryptosporidium hominis* was the most prevalent species and the most prevalent subtype family was Ib. Patients without diarrhea had significantly higher serum IgG levels to *Ch*gp15, *Ch*gp40 and *Cp*23, and higher fecal IgA levels to *Ch*gp15 and *Ch*gp40 than those with diarrhea suggesting that antibody responses to these antigens may be associated with protection from diarrhea and supporting further investigation of these antigens as vaccine candidates.

## INTRODUCTION

*Cryptosporidium* spp. are apicomplexan parasites that can cause severe, chronic diarrhea, wasting, and sometimes death in persons with untreated human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS), particularly in resource-constrained countries where antiretroviral therapy (ART) is not readily accessible or affordable.[1] Currently, there is no vaccine available, and the only U.S. Food and Drug Administration (FDA) approved drug for cryptosporidiosis, nitazoxanide, is not effective in immunocompromised hosts.[2,3] Nearly 6.2% of the adult population in Kenya lives with HIV/AIDS and a large proportion of those who are HIV-positive do not know that they are infected.[4] Among the ~1.5 million HIV infected adults, only about 400,000 (27%) are estimated to be on ART.[5] Diarrhea is a major cause of morbidity in HIV/AIDS patients and nearly 40% of those who die of AIDS experience diarrhea.[6,7] *Cryptosporidium* is the most common parasite identified in HIV/AIDS patients with diarrhea, and is reported to be the leading indicator of death among adult HIV/AIDS patients in Kenya.[6–11]

Both innate and adaptive immune responses play a role in protection from and resolution of cryptosporidiosis (reviewed in Reference 12). Cell-mediated immunity is crucial for resistance to and clearance of cryptosporidiosis (reviewed in Reference 12). The fact that AIDS patients are more susceptible to *Cryptosporidium* infections and the resolution of cryptosporidiosis following immune reconstitution underscores the importance of $CD4^+$ T cells.[13,14] Although the role of cell-mediated immunity in cryptosporidiosis is undisputable, humoral immune responses may also play a role (reviewed in References 12, 15, and 16). In human volunteer studies, serum antibody responses to *Cryptosporidium* were associated with resolution of infection or decreased severity of reinfection.[17–20]

The *Cryptosporidium* antigens gp15, gp40, and Cp23 (reviewed in References 12 and 21) are involved in attachment to and invasion of host cells. Serum antibodies to gp15 and Cp23 are associated with protection from diarrhea in immunocompetent adult human volunteers infected with *Cryptosporidium*.[16,17,20,22–25]

To date there have been no studies on the epidemiology of cryptosporidiosis in HIV/AIDS patients in Kenya, nor have there been any previously reported studies on the molecular characteristics of *Cryptosporidium* spp. infecting these patients or on immune responses to this parasite in this population. Therefore, the purpose of this study was to describe the epidemiological and clinical features of *Cryptosporidium* spp. infection in HIV/AIDS patients with and without diarrhea in Kenya, to investigate the species and subtype families of the infecting *Cryptosporidium* spp., and to determine antibody responses to *Cryptosporidium* antigens gp15, gp40, and Cp23 in these patients.

## MATERIALS AND METHODS

**Study site, population, and definitions.** This cross-sectional study was conducted at Kenyatta National Hospital (KNH), Nairobi, Kenya. KNH is the largest referral, teaching, and research hospital in Kenya with an average of 600,000 outpatient visits and 89,000 inpatients annually. About 200 HIV-positive patients are seen daily at KNH of which 50–80 are ART naive. Of these, about 10–15 patients are seen at the Comprehensive Care Clinic (CCC) of the Respiratory and Infectious Diseases Department, an outpatient facility that is involved in long-term care of HIV/AIDS patients from all over Kenya in accordance with the Kenya National AIDS/STI Control Program (NASCOP) guidelines. Ethics approvals for this study were obtained from the Kenya Medical Research Institute (KEMRI) Institutional Review Board, the Kenyatta National Hospital Ethical Review Committee, and the Tufts Medical Center Institutional Review Board. The

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study enrolled consecutive HIV-infected adults (18 years of age and older) presenting to CCC/KNH and who had not previously received ART.[26] Informed consent was obtained from all participants in the study.

Diarrhea was defined as three or more watery stools within a 24-hour period. A diarrheal episode was defined as diarrhea for at least 72 hours. The end of a diarrheal episode was defined as absence of diarrhea for 48 hours. Acute diarrhea was defined as a diarrheal episode lasting < 14 days. Persistent diarrhea was defined as a diarrheal episode lasting ≥ 14 days but < 30 days. Chronic diarrhea was defined as diarrhea lasting more than 30 days.

**Sample collection and testing.** A standardized questionnaire was used to collect sociodemographic data and prior medical history including known risk factors for cryptosporidiosis. At the time of enrollment a stool sample was obtained from each patient. Stool samples were tested by microscopy for ova and parasites and by polymerase chain reaction (PCR) at the 18S rRNA locus for *Cryptosporidium* spp.[27] Stool samples from patients with diarrhea were also tested by routine culture for enteric bacteria and by multiplex PCR for pathogenic *Escherichia coli*.[28] Blood was obtained from patients who were PCR positive for *Cryptosporidium* spp. and serum isolated. Serum and stool samples were stored at –80°C until further analysis.

**CD4 counts.** The $CD4^+$ T-cell counts were determined using a CyFlow SL3 (GmbH, Münster, Germany) at the Comprehensive Care Clinic at KNH.

***Cryptosporidium* species and subtype family determination.** The DNA was extracted from stool samples using a QIAamp Stool Mini kit (Qiagen Inc., Valencia, CA). Nested PCR followed by restriction fragment length polymorphism (RFLP) analysis at the 18S rRNA locus was used to determine the species and genotypes as described.[27] *Cryptosporidium* spp. subtype families were identified by PCR and RFLP analysis at the *gp40/15* locus (also known as $gp60$[29]) as previously described.[30]

**Recombinant antigens.** Sequences encoding gp15 and gp40 from *Cryptosporidium hominis* (TU502 isolate) and *Cryptosporidium parvum* (GCH1 isolate) genomic DNA, and a control protein containing only the fusion tags (Novagen, Madison, WI) cloned into the pET32/XaLIC vector (Novagen), were overexpressed in *E. coli* and purified by metal affinity chromatography as described.[31,32] A plasmid encoding Cp23 in the pGEX 4T-2 vector was obtained from Dr. Jeffrey Priest under a Materials Transfer Agreement from North Carolina State University, overexpressed in *E. coli*, the recombinant protein purified by glutathione–Sepharose affinity chromatography and the glutathione S transferase fusion tag removed by thrombin cleavage as previously described.[33]

**Serum enzyme-linked immunosorbent assays (ELISA).** Serum immunoglobulin G (IgG) and IgM responses to *Cryptosporidium* antigens were assessed by ELISA as previously described[34–36]; briefly, 96-well plates were coated overnight at 4°C with 0.5 μg/well of the following recombinant (r) *C. parvum* (Cp) and *C. hominis* (Ch) antigens in phosphate buffered saline (PBS): rCp23, rChgp15, rCpgp15, rChgp40, rCpgp40, or control antigen. Plates were washed three times with PBS, pH 7.2, containing 0.05% Tween 20 (PBST) and blocked with 0.25% bovine serum albumin (BSA) in PBS (BSA/PBS) for 2 hours at 37°C. Sera diluted 1:100 in 0.25% BSA/PBS were added to the wells and the plates incubated for 1 hour at 37°C, followed by washing three times with PBST. Alkaline phosphatase-conjugated goat anti-human IgG or IgM (Southern Biotech, Birmingham, AL) diluted in 0.25% BSA/PBS was added and the plates incubated for 1 hour at 37°C. After three washes with PBST, substrate solution containing *p*-nitrophenyl phosphate (Sigma, St. Louis, MO) at 1 mg/mL in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl2 was added and plates incubated for 30 minutes at room temperature in the dark.

Absorbance at 405 nm ($A_{405nm}$) was measured with a Bio-Rad microplate reader (model 550; Bio-Rad Laboratories, Hercules, CA). Known *Cryptosporidium*-negative and -positive (as determined by reactivity with *C. parvum* lysate by immunoblotting) serum samples were run on each plate. All samples were run in triplicate. The $A_{405nm}$ of the test sample for each antigen was subtracted from that for the control protein (to control for non-specific binding to the fusion tags). Plate-to-plate variation was normalized by dividing the $A_{405nm}$ of each sample by the $A_{405nm}$ of the positive control for that plate and multiplying by 100. Results were expressed as ELISA units.

**Fecal IgA ELISA.** One g of stool was diluted with 4 mL of PBST containing 100 μg/mL of soybean trypsin inhibitor (Calbiochem, San Diego, CA), 0.05M EDTA, and 10 mM phenylmethylsulfonyl fluoride (PMSF) (Calbiochem). The suspension was allowed to stand for 15 minutes at room temperature with intermittent shaking. The mixture was filtered through cheesecloth to remove particulate matter and centrifuged at 20,000 × *g* for 30 minutes. Total fecal IgA was determined by ELISA using purified human secretory IgA (AbD Serotec, Raleigh, NC) as standard and the samples adjusted to equivalent concentrations of sIgA. Fecal sIgA levels to each of the antigens were measured by ELISA using a modification of a previously described protocol[37]; briefly, 96-well plates were coated overnight at 4°C with 0.5 μg/well of the recombinant protein in 0.015M $Na_2CO_3$, 0.035M $NaHCO_3$, pH 9.6. Plates were washed three times with PBST and non-specific binding blocked with 5% fetal bovine serum (FBS) in PBST at room temperature for 5 hours. Fecal supernatants in 5% FBS in PBST were added and the plates incubated at 4°C overnight. Plates were washed three times with PBST, mouse monoclonal antibody to human IgA (clone GA-1, Sigma) diluted in 5% FBS in PBST was added and the plate incubated at 37°C for 1 hour. After washing three times with PBST, biotin-conjugated goat anti-mouse IgA (Southern Biotech) diluted in 5% FBS in PBST was added and the plate incubated at 37°C for 1 hour followed by three washes with PBST. Horseradish peroxidase conjugated to streptavidin (Thermo Scientific, Rockford, IL) diluted in 5% FBS in PBST was added and plates incubated at 37°C for 1 hour followed by three washes with PBST. The reaction was developed with substrate solution containing *ortho*-phenylenediamine (Thermo Scientific), 5 mg in 10 mL of 0.2 M sodium phosphate, 0.1 M citric acid pH 4.6, 4 μL of 30% hydrogen peroxide at room temperature, $A_{490}$ read after 20 minutes and ELISA units calculated as described previously.

**Statistical analysis.** Statistical analysis was performed using Graphpad Prism Version 6 (Graphpad, La Jolla, CA). Normally distributed continuous variables were reported as mean (SD) and compared using an unpaired *t* test with Welch's correction when variances were significantly

different. Non-normally distributed continuous variables were reported as median (interquartile range) and were compared using the Mann–Whitney test. Binary and categorical variables were presented as frequency (proportion) and were compared using the $\chi^2$ test with the exact method used when appropriate. The level of significance for all statistical analysis was two-sided $P < 0.05$.

## RESULTS

**Enteric pathogens.** Between June 2009 and July 2010 a total of 167 HIV-infected adults were consecutively enrolled in the study[26]; three patients did not provide samples and were therefore not included in the analysis. Demographics and clinical characteristics of the study population have been described previously.[26] Seventy of 164 (43%) of the patients had diarrhea and 94 of 164 (57%) did not have diarrhea. *Cryptosporidium* was detected in 17 of 164 (10%) of patients by microscopy and in 56 of 164 (34%) of patients by PCR. *Cryptosporidium* spp. were the most prevalent enteric pathogens and were identified in 56 of 164 (34%) of HIV/AIDS patients, including 25 of 70 (36%) patients with diarrhea and 31 of 94 (33%) patients without diarrhea. There was no significant difference in the prevalence of *Cryptosporidium* infection between patients with or without diarrhea. Eleven of 17 (64%) of the patients with microscopy-positive stools for *Cryptosporidium* had diarrhea compared with 25 of 56 (44%) of patients with PCR-positive stools. However, the difference was not statistically significant.

Nine of 25 (36%) of *Cryptosporidium* spp. positive patients with diarrhea and 13 of 31 (43%) without diarrhea were co-infected with other enteric parasites including *Cystoisospora belli* "syn *Isospora belli*," *Cyclospora cayetanensis*, *Giardia lamblia*, *Entamoeba histolytica*, *Ascaris lumbricoides*, *Schistosoma mansoni*, and *Ancylostoma duodenale*, with no significant difference in prevalence between the groups except for *A. lumbricoides*, which was only identified in patients without diarrhea. Enteric bacterial pathogens identified in *Cryptosporidium*-infected patients with diarrhea included enterotoxigenic *E. coli*, *Salmonella* spp., and *Klebsiella* spp. Four of 25 (16%) patients with diarrhea and 2 of 31 (6%) patients without diarrhea were co-infected with two or more pathogens other than *Cryptosporidium* spp.

**Patient groups.** Figure 1 shows the different patient groups in this study. There were 45 patients with diarrhea and enteric pathogens other than *Cryptosporidium* spp. detected. After excluding those patients with other enteric pathogens there were 16 patients with diarrhea and 18 patients without diarrhea who had only *Cryptosporidium* spp. identified in the stool. Analysis was therefore conducted on the following groups of patients, 1) 25 patients with diarrhea and *Cryptosporidium* spp. versus 45 patients with diarrhea and no *Cryptosporidium* spp., 2) 31 *Cryptosporidium*-infected patients without diarrhea versus 63 non-*Cryptosporidium*-infected patients without diarrhea, 3) 25 patients with diarrhea and enteric pathogens including *Cryptosporidium* spp. versus 31 patients without diarrhea but with enteric pathogens including *Cryptosporidium* spp., and 4) 16 patients with diarrhea and *Cryptosporidium* spp. but no other enteric pathogens versus 18 patients without diarrhea and *Cryptosporidium* spp. but no other enteric pathogens.

**Sociodemographic and epidemiological characteristics.** There were no significant differences in most sociodemographic characteristics between patients with or without diarrhea However, *Cryptosporidium*-infected patients with diarrhea had significantly more children per household than those without diarrhea ($P = 0.04$). Significantly more *Cryptosporidium*-infected patients with diarrhea reported contact with farm animals compared with patients with diarrhea but no *Cryptosporidium* infection (Table 1). Significantly more patients with diarrhea but without *Cryptosporidium* infection were infected with other enteric parasites ($P < 0.0001$) than those with *Cryptosporidium* infection (Table 1). Among the patients without diarrhea, there was a significantly higher percentage of married people with *Cryptosporidium* infection

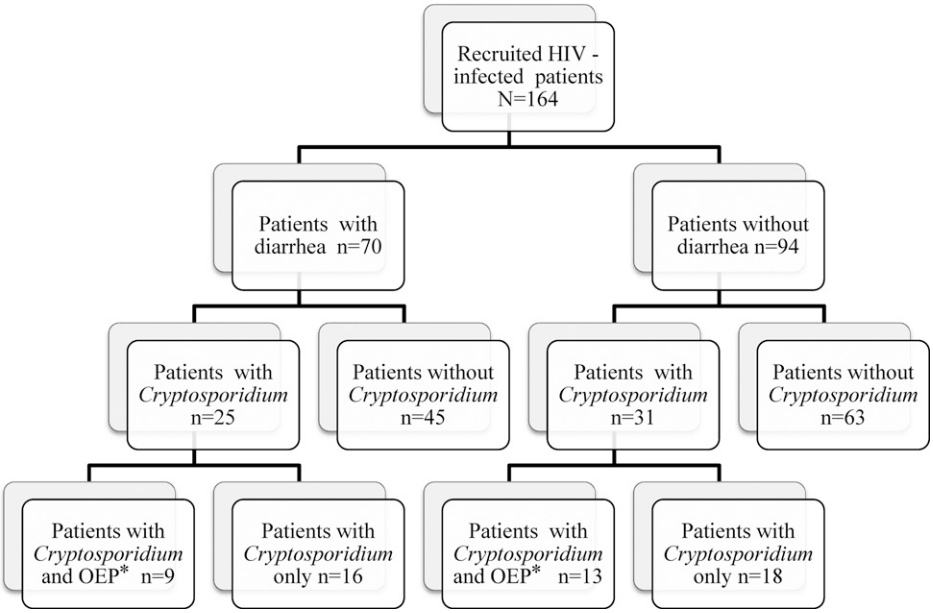


FIGURE 1. Schematic showing numbers of human immunodeficiency virus (HIV)-infected patients enrolled in the study with and without diarrhea and with and without *Cryptosporidium* infection. OEP = other enteric pathogens.

TABLE 1
Socio-demographic and epidemiological characteristics of HIV/AIDS patients with and without diarrhea and with and without *Cryptosporidium*

| Characteristic | Diarrhea | | | No diarrhea | | |
|---|---|---|---|---|---|---|
| | *Crypto* (*N* = 25) | No *Crypto* (*N* = 45) | *P* | *Crypto* (*N* = 31) | No *Crypto* (*N* = 63) | *P* |
| Age in years, mean ± SD | 34.40 ± 10 | 36.45 ± 9.0 | 0.49‡ | 39.5 ± 10 | 38.06 ± 10 | 0.99‡ |
| Male gender* | 9 (36) | 27 (60) | 0.08§ | 14 (45) | 19 (30) | 0.17§ |
| Married* | 9 (35) | 19 (42) | 0.06§ | 18 (58) | 22 (35) | **0.05**§ |
| Education level* | | | | | | |
| Primary | 7 (28) | 19 (42) | 0.31§ | 8 (26) | 13 (21) | 0.60§ |
| Secondary | 10 (40) | 21 (47) | 0.62§ | 15 (48) | 25 (40) | 0.51§ |
| High school | 9 (36) | 12 (27) | 0.42§ | 8 (26) | 15 (24) | 1.00§ |
| Monthly income†** | 30 (15, 36) | 29 (15, 43) | 0.52‖ | 20 (11, 50) | 30 (20, 42) | 0.29‖ |
| Number of adults† | 2 (1, 3) | 2 (2, 3) | 0.23‖ | 2 (2, 3) | 2 (2, 3) | 0.78‖ |
| Number of children† | 3 (2, 4) | 2 (1, 3) | **0.04**‖ | 2 (1, 3) | 3 (2, 3) | 0.64‖ |
| Permanent house* | 15 (60) | 23 (51) | 0.62§ | 18 (58) | 28 (44) | 0.27§ |
| Number of rooms† | 3 (2, 4) | 3 (2, 4) | 0.96¶ | 3 (2, 5) | 4 (3, 4) | 0.68¶ |
| Persons per room† | 2 (1, 2) | 2 (1, 2) | 0.27‖ | 1 (1, 2) | 1 (1, 2) | 0.24‖ |
| Contact dogs or cats* | 7 (28) | 11 (24) | 0.78§ | 13 (42) | 19 (30) | 0.35§ |
| Farm animals* | 15 (60) | 11 (24) | **0.005**§ | 7 (23) | 30 (48) | **0.03**§ |
| Poultry* | 10 (40) | 11 (24) | 0.19§ | 7 (23) | 25 (40) | 0.11§ |
| Tap water supply* | 19 (76) | 33 (73) | 1.00§ | 21 (68) | 50 (79) | 0.31§ |
| Water boiled/treated* | 9 (36) | 24 (53) | 0.22§ | 23 (74) | 50 (79) | 0.60§ |
| Other enteric parasites* | 9 (36) | 42 (93) | **0.0001**§ | 13 (43) | 40 (64) | 0.08§ |

*Crypto* = *Cryptosporidium*.
*Number (%).
†Median (interquartile range).
‡Unpaired *t* test.
§Fisher's exact test.
¶Chi-squared test using Fisher's exact test.
‖Mann–Whitney test.
**× 1,000 Kenyan shillings.
HIV = human immunodeficiency virus; AIDS = acquired immunodeficiency syndrome.

($P = 0.05$) than those without and significantly more patients ($P < 0.05$) reported contact with farm animals (Table 1). When we compared *Cryptosporidium*-infected patients who were infected with other enteric pathogens (Table 2), those with diarrhea were significantly younger ($P < 0.04$), had a significantly higher number of children per house hold ($P < 0.005$), and had a significantly higher number of individuals per room ($P < 0.04$) than those without diarrhea. A significantly higher ($P = 0.01$) percentage of patients with diarrhea reported contact with farm animals than those without diarrhea. Compared with patients with diarrhea, a significantly higher percentage of patients without diarrhea treated or boiled their drinking water ($P < 0.006$) (Table 2). When patients with enteric pathogens other than *Cryptosporidium* spp. were excluded, patients with diarrhea had a significantly higher number of children per household than those without

TABLE 2
Socio-demographic and epidemiological characteristics of *Cryptosporidium* spp.-infected HIV/AIDS patients with and without diarrhea

| Characteristics | Diarrhea (*N* = 25) | No diarrhea (*N* = 31) | *P* | Diarrhea NOEP (*N* = 16) | No diarrhea NOEP (*N* = 18) | *P* |
|---|---|---|---|---|---|---|
| Age in years, mean ± SD | 34.40 ± 10 | 39.5 ± 10 | **0.04**‡ | 34 ± 11 | 38 ± 10 | 0.26‡ |
| Male gender* | 9 (36) | 14 (45) | 0.59§ | 8 (50) | 7 (39) | 0.73§ |
| Married* | 8 (32) | 18 (58) | 0.06§ | 5 (69) | 10 (56) | 0.18§ |
| Education level* | | | | | | 0.71¶ |
| Primary | 6 (24) | 8 (26) | 1.00§ | 4 (25) | 4 (22) | |
| Secondary | 10 (40) | 15 (48) | 0.60§ | 7 (44) | 9 (50) | |
| High school | 9 (36) | 8 (26) | 0.39§ | 5 (31) | 5 (28) | |
| Monthly income†** | 30 (15, 36) | 20 (11, 50) | 0.87‖ | 21 (13, 34) | 16 (10, 28) | 0.62‖ |
| Number of adults† | 2 (1, 3) | 2 (2, 3) | 0.23‖ | 2 (1, 3) | 2 (1, 3) | 0.55‖ |
| Number of children† | 3 (2, 4) | 2 (1, 3) | **0.005**‖ | 3 (1, 4) | 2 (1, 3) | **0.004**‖ |
| Permanent house* | 15 (60) | 18 (58) | 1.00§ | 9 (56) | 11 (61) | 1.00§ |
| Number of rooms† | 3 (2, 4) | 3 (2, 5) | 0.79¶ | 4 (2, 4) | 3 (4, 5) | 0.94¶ |
| Persons per room† | 2 (1, 2) | 1 (1, 2) | **0.04**‖ | 1 (1, 2) | 1 (1, 2) | 0.34‖ |
| Contact dogs or cats* | 7 (28) | 13 (42) | 0.40§ | 6 (36) | 11 (61) | 0.30§ |
| Farm animals* | 15 (60) | 7 (23) | **0.01**§ | 10 (63) | 4 (29) | **0.03**§ |
| Poultry* | 10 (40) | 7 (23) | 0.24§ | 9 (56) | 4 (22) | 0.07§ |
| Tap water supply* | 19 (76) | 21 (68) | 0.56§ | 12 (75) | 11 (61) | 0.47§ |
| Water boiled/treated* | 9 (36) | 23 (74) | **0.006**§ | 6 (38) | 14 (78) | **0.03**§ |

NOEP = no other enteric pathogen detected.
*Number (%)
†Median (interquartile range).
‡Unpaired *t* test.
§Fisher's exact test.
¶Chi-squared test using Fisher's exact test.
‖Mann–Whitney test.
**× 1,000 Kenyan shillings.
HIV = human immunodeficiency virus; AIDS = acquired immunodeficiency syndrome.

TABLE 3
Clinical characteristics of *Cryptosporidium* spp.-infected HIV/AIDS patients

| Characteristics | Diarrhea ($N = 25$) | No diarrhea ($N = 31$) | $P$ | Diarrhea NOEP ($N = 16$) | No diarrhea NOEP ($N = 18$) | $P$ |
|---|---|---|---|---|---|---|
| Fever* | 13 (52) | 10 (32) | 0.18‡ | 8 (50) | 5 (28) | 0.29‡ |
| Vomiting* | 8 (32) | 0 (0) | **0.001**‡ | 4 (25) | 0 (0) | **0.04**‡ |
| Abdominal pain* | 19 (76) | 4 (13) | < **0.001**‡ | 10 (63) | 4 (22) | **0.03**‡ |
| Weight loss* | 20 (80) | 16 (52) | **0.05**‡ | 12 (75) | 7 (56) | **0.05**‡ |
| CD4 count (cells/mm$^3$)† | 108 (56, 268) | 229 (102, 337) | 0. 09§ | 120 (58, 330) | 207 (64, 413) | 0.40§ |
| CD4 cells < 200 (cells/mm$^3$)* | 15 (60) | 12 (39) | 0.18‡ | 10 (63) | 8 (44) | 0.33‡ |

NOEP = no other enteric pathogen.
*Number (%).
†Median (interquartile range).
‡Fisher's exact test.
§Mann–Whitney test.
HIV = human immunodeficiency virus; AIDS = acquired immunodeficiency syndrome.

diarrhea ($P < 0.005$), a significantly higher percentage of patients with diarrhea had contact with farm animals than those without diarrhea, although a significantly higher percentage of patients without diarrhea treated or boiled their drinking water ($P < 0.03$), compared with those with diarrhea (Table 2).

**Clinical characteristics.** Overall, 25 of 56 (45%) of *Cryptosporidium*-infected patients had diarrhea at the time of enrollment. Of these, 52% had acute diarrhea, 44% had persistent diarrhea, and 4% had chronic diarrhea. There was a significantly greater incidence of vomiting ($P < 0.001$), abdominal pain ($P < 0.0001$), and self-reported weight loss ($P < 0.05$) among *Cryptosporidium* spp.-infected patients with diarrhea compared with those without diarrhea. When patients with enteric pathogens other than *Cryptosporidium* spp. were excluded, the findings were similar. (Table 3). Comparison of *Cryptosporidium* spp.-infected patients with other enteric pathogens with diarrhea or without diarrhea revealed that there were no significant differences in the duration or type of diarrhea or number of diarrheal episodes in the past 6 or 12 months between them (not shown). There was no significant difference in $CD4^+$ T-cell counts or the number of patients with $CD4^+$ T-cell counts < 200 cells/mm$^2$ among *Cryptosporidium* spp.-infected patients with or without diarrhea (Table 3). Patients with persistent and chronic diarrhea had significantly lower $CD4^+$ T-cell counts compared with those with acute diarrhea (not shown).

***Cryptosporidium* species and subtype families.** *Cryptosporidium hominis* was the most common species identified and was detected in 34 (61%) patients, followed by *C. parvum* in 13 (23%), *Cryptosporidium canis* in 4 (7%), *Cryptosporidium meleagridis* in 3 (5%), and *Cryptosporidium suis* in 2 (4%) patients (Table 4). There were no significant differences in the prevalence of different species between patients with or without diarrhea (Table 4). This was the case regardless of the presence or absence of other enteric pathogens.

Overall, subtype family Ib was the most prevalent and was identified in 20% of patients (Table 4). Among patients with diarrhea, subtype family Ib was the most common and was identified in 7 patients, followed by Ie (3), IIa (3), IIe (3), If (2), and Id (1). Among the patients without diarrhea 1f was the most prevalent (5 patients) followed by Ie (4), Ib (3), Id (3), IIb (3), Ia (2), IIa (2), IIe (2), and Ic (1). We were not able to identify subtype families from two patients without diarrhea. There were no significant differences in the incidence of different subtype families among patients with or without diarrhea regardless of the presence or absence of other parasites.

**Antibody responses to immunodominant and polymorphic *Cryptosporidium* spp. antigens.** We investigated serum IgG and IgM and fecal IgA responses to the immunodominant, conserved Cp23 antigen from *C. parvum* and the immunodominant, but less conserved gp15 antigen from *C. parvum* (*Cp*gp15), and *C. hominis* (*Ch*gp15) and the polymorphic gp40 antigen from both species (*Cp*gp40 and *Ch*gp40). Because antibody responses may be influenced by co-infection with other pathogens, we only report antibody responses in those who were infected exclusively with *Cryptosporidium*.

Interestingly, patients without diarrhea had significantly higher levels of serum anti-*Ch*gp15 IgG ($P < 0.05$), anti-*Ch*gp40 IgG ($P < 0.05$), and anti-Cp23 serum IgG ($P < 0.05$) compared with those with diarrhea (Figure 2). There were no significant differences in the IgG levels to *Cp*gp15 and *Cp*gp40 between patients with and without diarrhea (not shown). Patients with diarrhea also had significantly higher levels of anti-*Ch*gp40 IgM ($P < 0.05$) compared with those without diarrhea (not shown). There was no significant difference in IgM levels to any of the other *Cryptosporidium* antigens between patients with and without diarrhea (not shown). Figure 3 shows that patients without diarrhea had significantly higher fecal IgA levels of anti-*Ch*gp15 IgA ($P < 0.005$) and anti-*Ch*gp40 IgA ($P < 0.05$); however, there were no significant differences in IgA levels to *Cp*gp15, *Cp*gp40, or Cp23 among patients with or without diarrhea (not shown). Taken together, these results suggest that

TABLE 4
*Cryptosporidium* species and subtype families identified in HIV/AIDS patients*

| Species | Diarrhea ($N = 25$) | No diarrhea ($N = 31$) | $P$ |
|---|---|---|---|
| *C. hominis* | 14 (56) | 20 (65) | 0.59 |
| *C. parvum* | 6 (24) | 7 (23) | 1.00 |
| *C. meleagridis* | 2 (8) | 1 (3) | 0.58 |
| *C. canis* | 2 (8) | 2 (6) | 1.00 |
| *C. suis* | 1 (4) | 1 (3) | 1.00 |
| **Subtypes** | **Diarrhea ($N = 20$)** | **No diarrhea ($N = 25$)** | **$P$** |
| Ia | 2 (10) | 2 (8) | 1.00 |
| Ib | 6 (30) | 3 (12) | 0.15 |
| Id | 1 (5) | 3 (12) | 0.62 |
| Ie | 3 (15) | 4 (18) | 1.00 |
| If | 2 (10) | 5 (20) | 1.00 |
| IIa | 3 (15) | 2 (8) | 0.64 |
| IIa | 3 (15) | 2 (8) | 0.64 |
| IIb | 0 (0) | 3 (12) | 0.24 |
| IIe | 3 (15) | 2 (8) | 0.64 |
| IIc | 0 (0) | 1 (4) | 1.00 |

*All data are shown as number (%). Fisher's exact test was used for analysis.
HIV = human immunodeficiency virus; AIDS = acquired immunodeficiency syndrome.

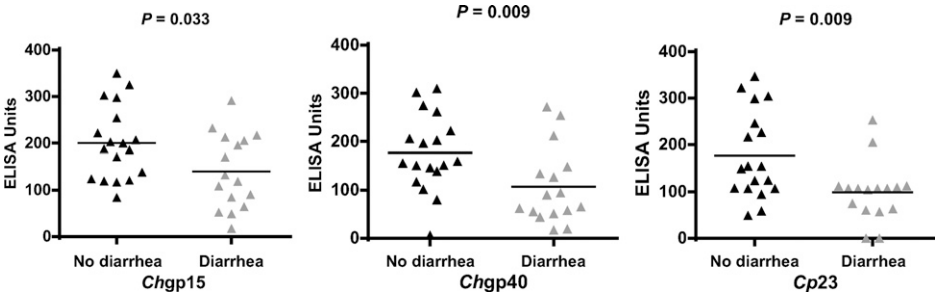


FIGURE 2. Serum immunoglobulin G (IgG) antibodies to *Ch*gp15, *Ch*gp40, and *Cp*23: Serum IgG antibodies to specific antigens in *Cryptosporidium* spp.-infected human immunodeficiency virus (HIV)-positive patients with or without diarrhea were measured by enzyme-linked immunosorbent assay (ELISA) as described in the Materials and Methods.

serum and fecal antibodies to *C. hominis* gp15 and gp40 and serum antibodies to Cp23 may be associated with protection from diarrheal symptoms.

## DISCUSSION

This is the first study comparing cryptosporidiosis in HIV/AIDS patients with and without diarrhea and the first study of immune responses to *Cryptosporidium* antigens in Kenya. In this study *Cryptosporidium* spp. were the most prevalent gastrointestinal pathogens and were identified in about a third of patients, regardless of whether they had diarrhea or not. Our data confirms previous reports that *Cryptosporidium* spp. infection is common among HIV-infected adults in Kenya.[6–9,11] The prevalence of *Cryptosporidium* spp. in adults in this study (34%) is higher than that reported by Gatei and others[10] in HIV-infected children < 5 years of age (4%) in Kenya. However, in this study we used PCR to detect *Cryptosporidium* spp., whereas Gatei and others used microscopy. This study also confirms previous studies that have shown that PCR is more sensitive than microscopy in *Cryptosporidium* spp. detection.[38,39]

Of particular interest in this study is the high rate of asymptomatic (without diarrhea) cryptosporidiosis. This is a higher rate of asymptomatic cryptosporidiosis than has been reported so far in Africa.[40–43] As Houpt and others noted, previous reports may have underestimated the rates of asymptomatic infections as few HIV/AIDS patients without diarrhea have been tested for *Cryptosporidium* spp.[41] In other resource-constrained countries higher rates of asymptomatic cryptosporidiosis have been reported, even among patients with low $CD4^+$ T-cell counts.[44–46] The basis for the high rates of asymptomatic cryptosporidiosis in HIV/AIDS patients despite low $CD4^+$ T-cell counts is unknown. It is possible that asymptomatic cryptosporidiosis could be associated with low parasite loads, although no significant correlations were found between parasite loads and diarrheal symptoms in a study in Bangladesh.[47] In our study there was no statistically significant difference in the occurrence of diarrhea between those who had microscopy-positive stools for *Cryptosporidium* compared with those who had PCR-positive stools. However, in resource-constrained countries such as Kenya where cryptosporidiosis is endemic, children are exposed early and frequently to *Cryptosporidium* spp. Pre-existing antibody and/or T cell memory immune responses to *Cryptosporidium* spp. infection acquired before HIV infection may protect from subsequent symptomatic cryptosporidiosis in these patients.

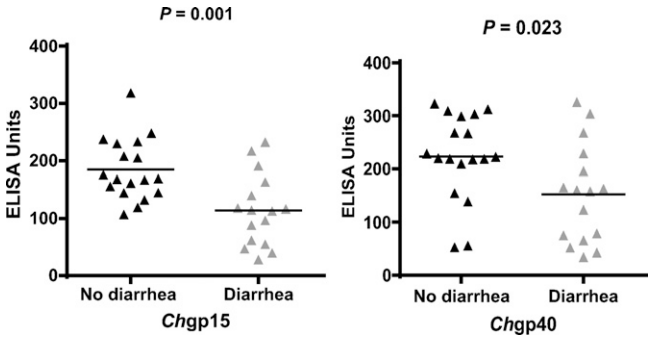


FIGURE 3. Fecal immunoglobulin A (IgA) antibodies to *Ch*gp15 and *Ch*gp40: Fecal IgA antibodies to specific antigens in *Cryptosporidium* spp.-infected human immunodeficiency virus (HIV)-positive patients with or without diarrhea were measured by enzyme-linked immunosorbent assay (ELISA) as described in the Materials and Methods.

*Cryptosporidium* spp.-infected patients with diarrhea were significantly younger and lived in more crowded houses with more children than those without diarrhea. In addition, those with diarrhea reported significantly more contact with farm animals and were less likely to treat or boil their drinking water than those without diarrhea. Although all of these are known risk factors for *Cryptosporidium* spp. infection,[48] the reasons for their association with diarrhea are not clear but may be related to a higher parasite burden in patients with diarrhea compared with those without. *Cryptosporidium*, which frequently infects children, could be transmitted from the children to adults in these crowded homes. As for patients with diarrhea being younger, previous longitudinal and population-based cross-sectional studies have shown that the prevalence and magnitude of the serum antibody response to specific *Cryptosporidium* antigens increases with increasing age and infection experience.[25,49] As expected, *Cryptosporidium* spp.-infected patients with diarrhea also had significantly higher rates of other symptoms such as abdominal pain and weight loss (self-reported) than those without diarrhea.

The distribution of *Cryptosporidium* species varies from country to country and even from one region to another[50]; the majority of the patients in this study were infected with *C. hominis* followed by *C. parvum* species. This is consistent with a previous study on HIV-infected children in Kenya, in whom *C. hominis* and *C. parvum* were the main species

identified with prevalence rates of 87% and 9%, respectively.[10] Studies from other resource-constrained countries have also reported *C. hominis*, followed by *C. parvum* as the most prevalent species in HIV-infected patients.[41,51–54] *C. hominis* transmission is anthroponotic (human-to-human) either directly or indirectly through contaminated water or food, whereas *C. parvum* transmission can be anthroponotic or zoonotic (acquired from infected animals).[29]

A few patients were infected with zoonotically transmitted species including *C. canis* (7%), *C. meleagridis* (5%), and *C. suis* (3%). *C. meleagridis*, *C. canis*, and *C. muris* infections have also been previously reported in HIV-infected patients in Kenya.[10,52,55,56] Thus far *C. suis* has not been reported in Kenya but has been reported in HIV-infected adults in Peru.[51] In our study, 72% of patients with diarrhea and 55% of those without diarrhea reported having contact with farm animals, domestic pets, or poultry suggesting that they may have acquired infection with zoonotically transmitted species from them. A previous study reported that *C. hominis* infection was associated with a higher rate of asymptomatic infection and a lower $CD4^+$ T-cell count in Tanzanian patients with HIV when compared with *C. parvum* infection.[41] However, in our study there were no significant differences between the species infecting patients with or without diarrhea.

Sequence analysis at the *Cpgp40/15* (*gp60*) locus has allowed the identification of at least seven subtype families in *C. hominis* isolates and 11 in *C. parvum*.[29,57] In our study, we identified five *C. hominis* subtype families, the most common being Ib and four *C. parvum* subtype families, the most frequent being IIa and IIe. *Cryptosporidium hominis* subtype family Ib is reported to be the most common in the world representing about half of all subtype families identified in humans.[57] *Cryptosporidium parvum* subtype family IIa is transmitted zoonotically, whereas transmission of subtype family IIe is anthroponotic.[29,57] The only previously available data on *Cryptosporidium* spp. subtype families in Kenya are from six human diarrheal samples (HIV status unknown) in which two contained subtype Ib and four contained subtype IIb.[58] In our study there were no significant differences in the subtype families infecting patients with or without diarrhea. Studies from India and Peru have shown that HIV-infected patients are infected with a greater diversity of species and subtypes compared with immunocompetent individuals.[38,51,53] *Cryptosporidium* isolates from Ugandan children with and without HIV infection also displayed extensive diversity (8 subtype families in 28 isolates) at the *gp40/15* (GP60) locus.[59]

There have been no previous studies from Kenya on immune responses to *Cryptosporidium*. We investigated serum and mucosal antibody responses to gp40, gp15, and Cp23 in HIV-infected patients with *Cryptosporidium*. The gp40 and gp15 are proteolytic cleavage products of gp40/15, a major surface glycoprotein of *Cryptosporidium*.[31,60] The gp40 is the N-terminal cleavage product, whereas gp15 is the C-terminal product.[31] Both of these antigens are implicated in attachment to and invasion of host cells.[31,60] The presence of preexisting antibodies to gp15 was associated with protection from diarrheal symptoms in infected adult humans.[20,23] However, although antibody responses to gp40 have been reported,[34] it is not known if these responses are associated with protection from symptoms.

The Cp23 is an immunodominant antigen found on the surface of the invasive stages of *Cryptosporidium* and is shed from their surface during their gliding motility[61–63]; in a human volunteer study, pre-existing antibodies to Cp23 were associated with a reduction in oocyst shedding in infected volunteers.[20]

In our study, IgG responses to *C. hominis* antigens (*Ch*rgp15 and *Ch*rgp40) but not the corresponding C. *parvum* antigens were significantly higher in patients without diarrhea. Because most patients in this study were infected with *C. hominis,* this suggests that responses to the homotypic antigens may be associated with protection from diarrhea. The gp40 is highly polymorphic among *C. parvum* and *C. hominis* isolates,[64,65] which is consistent with the possibility that protection from diarrhea is associated with responses to the homotypic antigen and with the results of a study in India in which we found that *C. hominis* gp40 induced higher IgG responses than *C. parvum* gp40 in infected children.[34] Although there are polymorphisms among *C. parvum* and *C. hominis* gp15,[35] this antigen is relatively conserved between both species. Our previous studies in Bangladesh indicated that there is significant cross-reactivity between them.[35] It is therefore interesting to note that responses to the homotypic antigen were associated with protection from diarrhea. Antibody responses to the Cp23 antigen, which is highly conserved among *C. parvum* or *C. hominis* isolates,[36,66] were also associated with protection from diarrhea.

Although most studies on antibody responses in cryptosporidiosis have been carried out in presumably immunocompetent individuals, a few studies have also reported *Cryptosporidium*-specific antibody responses in HIV-infected patients. Ungar and others[67] showed that patients with and without AIDS have serum IgG, IgM, and IgA responses to *Cryptosporidium*. In HIV-infected patients, a strong serological response to the 27-kDa *Cryptosporidium* antigen (same as Cp23) was associated with a reduced risk of diarrhea.[23] A retrospective cohort study carried out on HIV-infected men showed antibody responses to both the recombinant 27-kDa (Cp23) and the native 17-kDa (gp15) antigens in response to *Cryptosporidium* infection.[68] A positive IgG and IgA response to crude soluble *C. parvum* antigen, was observed in a significantly higher number of *Cryptosporidium*-infected individuals compared with *Cryptosporidium* uninfected individuals in both HIV-infected as well as uninfected individuals.[69]

Our study suggests that antibody responses to specific antigens are associated with protection from diarrhea in *Cryptosporidium*-infected HIV/AIDS patients. However, there are reports of diarrhea in patients with AIDS despite the development of *Cryptosporidium*-specific antibodies,[70] and others have suggested that *Cryptosporidium*-specific antibody responses may not be necessarily associated with protection from cryptosporidiosis.[69]

Clearly, cell-mediated responses play a major role in protection from and resolution of *Cryptosporidium* infection. It is well known that HIV/AIDS patients with lower CD4 counts are more susceptible to cryptosporidiosis and have greater severity of disease[71]; in our study, we were not able to assess cell-mediated responses to specific antigens. However, it is important to stress that although antibody responses to specific antigens were associated with protection from diarrhea, it remains to be determined whether these responses, particularly those that are T cell-dependent are themselves

protective or whether they are markers of underlying protective cell-mediated responses.[72] The finding that IgG responses to specific antigens were associated with protection from diarrhea suggests that they are T-cell dependent and raise the possibility that they reflect underlying T cell-mediated responses to these antigens. It is also important to note that other factors such as host genetics and malnutrition could also account for low antibody levels in HIV-infected patients with diarrhea.[73]

Our study is the first to report fecal IgA responses to specific *Cryptosporidium* antigens. A previous study using oocysts as the antigen found that *Cryptosporidium*-specific fecal IgA antibody responses in human volunteers correlated significantly with the presence of active or recent infection.[74]

There were several limitations to this study. The numbers of subjects in each group were small. Of importance, we were not able to assess cell mediated immune responses to any of the antigens. We were not able to assess the presence of enteric viruses in any patient or bacterial pathogens in patients without diarrhea. We were also not able to use sensitive molecular methods for detection of other pathogens. Recently, Mejia and others[75] showed an increased sensitivity of real time quantitative PCR for protozoans and helminthes compared with microscopy. *Cryptosporidium* was detected by both microscopy and conventional PCR, whereas the other enteric parasites were detected with microscopy alone. We may therefore have underestimated the prevalence of these other pathogens in this population. Co-infection with pathogens such as helminthes may cause immunomodulation that may influence the manifestation of diarrhea and or the host immune responses to *Cryptosporidium* spp.[76,77] Because of the small sample size and the semi-quantitative nature of the ELISA we used, we were not able to evaluate whether antibody levels to species-specific antigens were associated with the infecting *Cryptosporidium* spp. Finally, this was a cross-sectional study and we were not able to assess previous exposure to *Cryptosporidium* spp., persistence of antibodies over time, or antibody responses following ART. However, despite all these limitations, the results of our study stress the importance of *Cryptosporidium* as a major pathogen in untreated HIV/AIDS patients in Kenya, suggest that antibody responses to specific antigens are associated with protection from diarrhea in *Cryptosporidium*-infected patients, and support further investigation of gp15, gp40, and Cp23 as putative vaccine candidates. Future studies with larger numbers of patients with and without diarrhea, before and after ART, and with assessment of effector and memory T cell responses are needed to evaluate the potential of these antigens as putative components of subunit vaccine development for vulnerable populations in resource-constrained areas.

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