

Original Article

***Cryptosporidium* species and subtypes in diarrheal children and HIV-infected persons in Ebonyi and Nsukka, Nigeria**

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Abstract

Introduction: Cryptosporidiosis is a common disease of children and immune-compromised persons. This study evaluated the diversity and distribution of *Cryptosporidium* species in diarrheal children and HIV-infected persons on highly active antiretroviral therapy (HAART) and those not on HAART.

Methodology: A total of 394 fecal specimens were collected from patients attending clinics in Nsukka and Ebonyi, Nigeria. Detection and identification of *Cryptosporidium* species were conducted by PCR-RFLP of the small subunit (SSU) rRNA gene, whereas subtyping was done by sequence analysis of the 60 kDa glycoprotein (gp60) gene.

Results: Twenty-five (6.3%) specimens yielded four *Cryptosporidium* species, including *C. hominis*, *C. parvum*, *C. felis*, and *C. viatorum*. *C. hominis* was the most dominant species with 48.0% occurrence and three identified subtype families: Ia (six specimens), Ib (three specimens), Ie (two specimens), and one un-subtyped species. *C. parvum* had 44.0% occurrence and two subtype families: IIc (eight specimens) and IIe (three specimens), while *C. felis* and *C. viatorum* each had 4.0% occurrence. There were significant differences in *Cryptosporidium* species distribution between age groups in children and HIV-infected persons, between suburban and urban areas, and between low and high CD4⁺ cell counts in HIV-infected patients. There were no significant differences in infection rate and species distribution between HIV-infected patients on HAART and those not on HAART.

Conclusions: The results from this study show that there is a high diversity of *Cryptosporidium* spp. in humans in Ebonyi and Nsukka, Nigeria, and that all the *C. parvum* subtypes identified are most likely anthroponotic in origin.

Key words: *Cryptosporidium*; genetic diversity; HAART; HIV-infected persons; diarrheal children

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Introduction

Cryptosporidium is a protozoan parasite that has been implicated in diarrheal illness in children and immune-compromised individuals such as HIV-infected persons [1,2]. The parasite can be zoonotic or anthroponotic in origin and has been implicated in diarrhea outbreaks in different parts of the world [3-5]. Infections in HIV⁺ patients have been associated with low CD4⁺ cell counts (< 200 cells/mm³), lack of access to highly active antiretroviral therapy (HAART), and poor hygiene, while infections in children are associated mostly with young age and poor hygiene [4,6,7].

The use of molecular diagnostic tools has greatly enhanced the identification of infection sources and transmission routes of *Cryptosporidium* [3].

Genotyping and subtyping of *Cryptosporidium* species are done mostly by polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) analysis of the small subunit (SSU) rRNA gene and the sequencing of the 60 kDa glycoprotein gene (gp60). This has led to identification of about 20 *Cryptosporidium* species and genotypes in humans, including *C. parvum*, *C. hominis*, *C. meleagridis*, *C. canis*, *C. felis*, and the recently identified *C. viatorum*. Among them, *C. parvum* and *C. hominis* are most common. These *Cryptosporidium* species have been found in both HIV⁺ patients and children.

Molecular characterizations of *Cryptosporidium* spp. in a small number of HIV-infected persons and children in southwestern and mid-western states have provided some information on *Cryptosporidium* species

and subtypes in Nigeria [4,6,8-11]. However, no information is available on the species and subtypes circulating in humans in south-eastern Nigeria. In this study, a molecular epidemiological study of *Cryptosporidium* spp. in HIV-infected patients and diarrheal children in Ebonyi and Nsukka, south-eastern Nigeria, was conducted.

Methodology

Specimens

Fecal specimens were collected from 394 patients including diarrheal children and HIV- positive individuals attending clinics at 7 healthcare facilities: Mile Four Hospital and Jesus is Lord Clinic in Abakaliki urban area of Ebonyi state; Mater Hospital, Afikpo (southern suburban area of Ebonyi state); General Hospital, Ezzamgbo (northern suburban area of Ebonyi state); Okposi Clinic and Godspower Clinic in Ebonyi central suburban area; and Bishop Shanahan Hospital, Nsukka, Enugu state. Both Ebonyi and Enugu states are in southeastern Nigeria. The Ebonyi state clinics are located in urban and suburban areas, while the Bishop Shanahan Hospital in Nsukka serves suburban residents. Of the 394 patients sampled, 143 were diarrheal children under 6 years of age from urban and suburban areas of Ebonyi state, while 251 were HIV-infected persons with diarrhea or related illness from Ebonyi (n = 115) and Nsukka (n = 136). Data on CD4⁺ cell counts, history of HAART in HIV⁺ persons were obtained from each patient. Ethical permission and informed consent were obtained in each hospital/clinic where specimens were collected. The study protocols were approved by the hospital's research ethics committees in line with the Nigerian Ministry of Health guidelines. The fecal specimens were preserved in 2.5% potassium dichromate at 4°C and shipped to the Centers for Disease Control and Prevention (CDC), Atlanta, USA for molecular analysis. Information on patients' personal details was kept strictly confidential.

DNA extraction

The fecal specimens were washed twice with distilled water by centrifugation. DNA was extracted from 0.2 mL of the concentrates using the FastDNA SPIN kit for soil (MP Biologicals, Irvine, USA). The extracted DNA was stored at -20°C prior to polymerase chain reaction (PCR) analysis.

PCR detection of *Cryptosporidium* species and RFLP analysis

The extracted DNA was analyzed for *Cryptosporidium* spp. by nested PCR analysis of the SSU rRNA gene according to Xiao *et al.* [12]. RFLP analysis of the secondary PCR products using restriction enzymes *SspI* (New England Biolab, Ipswich, USA) and *VspI* (Promega, Madison, USA) was used to identify *Cryptosporidium* species. DNA sequencing was used to confirm the diagnosis of *C. felis* and *C. viatorum*. Subtyping was done by DNA sequence analysis of the gp60 gene as described by Alves *et al.* [13].

Sequence analysis

DNA sequencing of the secondary PCR products of SSU rRNA and gp60 genes was carried out using the BigDye Terminator Sequencing Kit (Applied Biosystems, Foster City, USA) and an ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Foster City, USA). The nucleotide sequences were assembled and edited using ChromasPro version 1.7.5 software (Technelysium, South Brisbane, Australia). To confirm the species identification and subtypes, the consensus sequences were aligned with each other and reference sequences of each gene downloaded from GenBank using ClustalX (<http://www.clustal.org/>). *Cryptosporidium* subtypes were named based on the established naming terminology [3].

Statistical analysis

The association of *Cryptosporidium* infections with CD4⁺ cell counts and HAART history in HIV-infected persons was determined by χ^2 analysis. The difference in infection rates between urban and sub-urban areas or between HIV-infected patients in Ebonyi, Nsukka and diarrheal children were evaluated using one-way ANOVA. All statistical analyses were performed using SPSS version 16 at the 95% confidence level.

Results

Occurrence of *Cryptosporidium* spp. in HIV⁺ persons and diarrheal children in Ebonyi and Nsukka

A total of 25 of the 394 specimens screened in this study were positive for *Cryptosporidium* spp., giving a general prevalence of 6.3%. The 25 positive samples comprised 20 of 258 samples examined in Ebonyi and 5 of 136 samples from Nsukka, thus giving prevalence values of 7.8% and 3.7% for Ebonyi and Nsukka, respectively. Within Ebonyi, there was a significantly ($p < 0.05$) higher prevalence of *Cryptosporidium* in the suburban area (13.6%) than in the urban area (4.7%).

Of the 394 samples, 251 were from HIV⁺ persons, while 143 were from children. Infection rates in the HIV⁺ persons and children were 6.8% and 5.6%, respectively. All the infected children were under 2 years of age, and no infection was observed in older children (Figure 1). Among the HIV⁺ population, infection rates were higher in Ebonyi (4.7%) than in Nsukka (3.7%).

Occurrence of Cryptosporidium spp. in HIV⁺ patients of different age groups and CD4⁺ cell counts

The highest *Cryptosporidium* infection rate (18.8%) was observed in HIV⁺ patients over 50 years of age, followed by a 14.3% infection rate in the 0–10 years age group, while the lowest rate (1.4%) was observed among the 21–30 years age group (Figure 2). Based on CD4⁺ cell counts, the infection rate in HIV⁺ patients with CD4⁺ cell counts < 200 cells/mm³ (19.1%) was significantly ($p < 0.05$) higher than in those with CD4⁺ cell counts > 200 cells/mm³ (7.9%) in Ebonyi. Similarly, HIV⁺ patients in Nsukka with CD4⁺ cell counts < 200 cells/mm³ had higher infection rates (5.9%) than those with CD4⁺ cell counts >200 cells/mm³ (3.4%) (Figure 3). Altogether, of the 251 HIV-infected persons sampled in this study, 9.7% (7/72) of patients with CD4⁺ cell counts < 200 cells/mm³ had *Cryptosporidium*, whereas 5.2% (9/174) of patients with CD4⁺ cell counts > 200 cells/mm³ were infected.

Occurrence of Cryptosporidium infection in HIV⁺ patients receiving HAART and those not receiving HAART

Review of patients’ clinical histories showed that 47 of 115 HIV⁺ patients in Ebonyi were on HAART, while 68 were not. Of the 47 patients on HAART, 5 (10.6%) were infected with *Cryptosporidium* as opposed to 7 of 68 (10.3%) patients who were not on HAART. Similarly, in Nsukka, 3 of 67 (4.5%) HIV⁺ patients on HAART were infected, while 2 of 69 (2.9%) of patients not on HAART were infected (Figure 4). Therefore, a total of 8/113 or 7.1% of HIV-infected persons on HAART were co-infected with *Cryptosporidium*, while 9/138 or 6.5% of non-HAART patients were co-infected. These differences were not statistically significant ($p > 0.05$). Of the 8 patients who were on HAART and infected with *Cryptosporidium*, 7 were residents of suburban areas, while 1 patient was a resident of an urban area.

Figure 1. Occurrence of *Cryptosporidium* in children of different age groups. The bars in the figure represent percentage occurrence of *Cryptosporidium* spp. in diarrheal children under one month and those greater than 48 months of age.

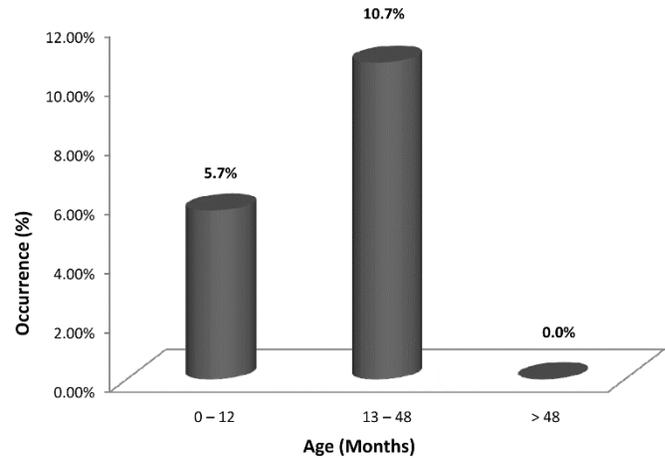


Figure 2. Occurrence of *Cryptosporidium* in HIV⁺ patients of different age groups. The bars in the figure represent percentage occurrence of *Cryptosporidium* spp. in HIV⁺ patients of different age groups in the study areas.

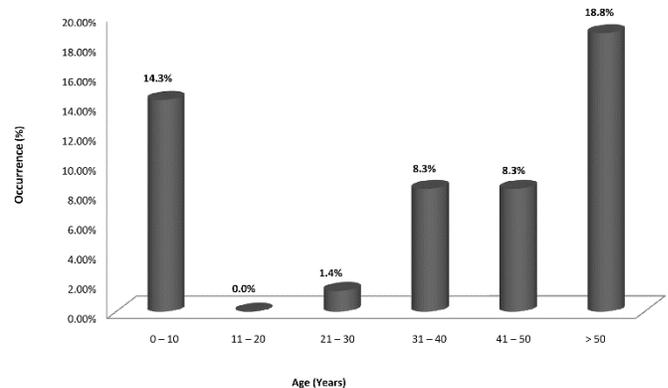
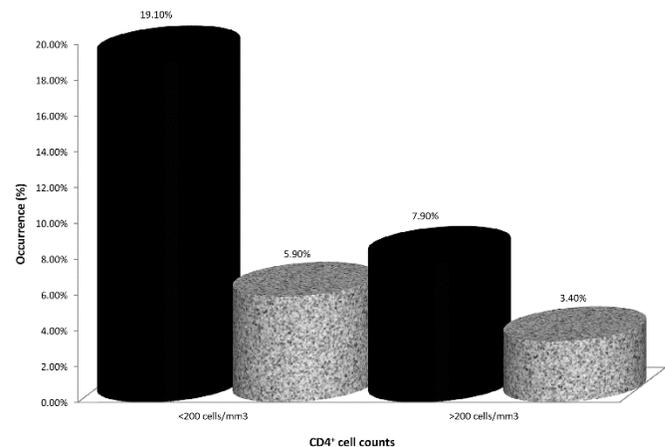


Figure 3. Occurrence of *Cryptosporidium* in HIV⁺ patients of different CD4⁺ cell counts. The black and grey bars in the figure represent percentage occurrence of *Cryptosporidium* spp. in HIV⁺ patients of different CD4⁺ cell counts at different study locations (Nsukka and Ebonyi respectively). The two bars (left) represent those with CD4⁺ < 200 cells/mm³, whereas the two right bars represent those with CD4⁺ > 200 cells/mm³.



Distribution of Cryptosporidium species and subtypes among specimens

Four *Cryptosporidium* species – *C. hominis*, *C. parvum*, *C. felis*, and *C. viatorum* – were identified among the 25 *Cryptosporidium*-positive specimens. In Ebonyi, *C. parvum* was the dominant species in suburban areas (8/12), while *C. hominis* was the dominant species in urban areas (7/8). A higher diversity of *Cryptosporidium* species was seen in HIV+ patients in Nsukka; although only five specimens were positive, they belonged to *C. parvum* (2), *C. hominis* (1), *C. felis* (1), and *C. viatorum* (1) (Table 1).

A total of 10 *Cryptosporidium* subtypes were identified, including 7 *C. hominis* subtypes (IaA18R3, IaA25R3, IaA27R4, IaA29R3, IbA10G2, IbA13G3, and IeA11G3T3) and 3 *C. parvum* subtypes (IleA10G1, IicA5G3b and IicA5G3k). One *C. hominis*, which could not be matched with any known subtype, was also identified from Ebonyi (Table 1). Among the *C. hominis* subtypes, IaA18R3 had the highest occurrence, followed by IbA13G3, with 3 and 2 positive samples

Figure 4. Occurrence of *Cryptosporidium* in HIV+ patients on HAART and not on HAART. The black and grey bars in the figure represent percentage occurrence of *Cryptosporidium* spp. in HIV+ patients placed on HAART and those not placed HAART therapy at different study locations (Nsukka and Ebonyi respectively). The two bars on the left represent those placed on HAART therapy, whereas the two right bars represent those who were not placed on HAART in the study locations.

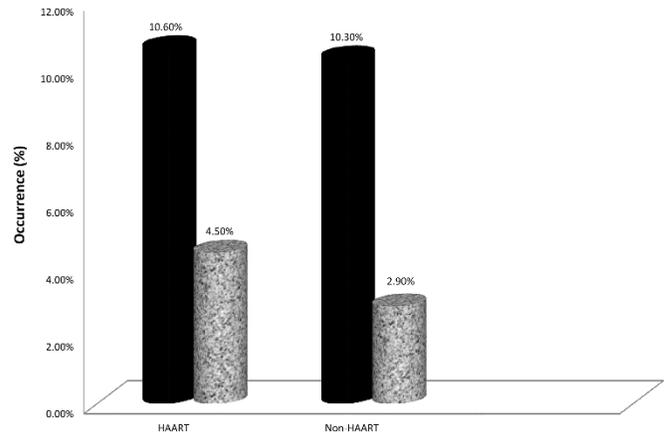


Table 1. Distribution of *Cryptosporidium* species and subtypes in diarrheal children and HIV+ patients in Ebonyi and Nsukka, Nigeria

Location	Sample population	No of specimens infected (%)	Species and number positive	Subtype family	Subtypes	Number positive	
Ebonyi Suburban	HIV+ (n = 61)	9 (14.8%)	<i>C. parvum</i> (6)	Iic	IicA5G3b	3	
				Iie	IieA10G1	3	
				Ia	IaA27R4	1	
				Ie	IeA11G3T3	1	
				Un-subtyped		1	
	Diarrheal children (n = 27)	3 (11.1%)	<i>C. parvum</i> (2)	Iic	IicA5G3b	2	
Ebonyi Urban	HIV+ (n = 54)	3 (5.6%)	<i>C. hominis</i> (3)	Ia	IaA18R3	2	
				Ib	IbA10G2	1	
	Diarrheal children (n = 116)	5 (4.3%)	<i>C. parvum</i> (1)	Iic	IicA5G3k	1	
				<i>C. hominis</i> (4)	Ia	IaA18R3	1
					Ia	IaA25R3	1
					Ib	IbA13G3	1
					Ie	IeA11G3T3	1
					Un-subtyped		1
Nsukka	HIV+ (n = 136)	5 (3.7%)	<i>C. parvum</i> (2)	Iic	IicA5G3k	2	
				Ia	IaA29R3	1	
			<i>C. hominis</i> (1)				
				<i>C. felis</i> (1)			
<i>C. viatorum</i> (1)							

HIV+: human immunodeficiency virus-infected person; IicA5G3b: *Cryptosporidium parvum* with 5 repeats of TCA, 3 repeats of TCG and 2 SNPs; IicA5G3k: *Cryptosporidium parvum* with 5 repeats of TCA, 3 repeats of TCG and 11 SNPs; IieA10G1: *Cryptosporidium parvum* with 10 repeats of TCA, one TCG at repeat region; IaA27R4: *Cryptosporidium hominis* with 27 repeats of TCA and 4 other repeats of 13/15 base pairs; IaA18R3: *Cryptosporidium hominis* with 18 repeats of TCA and 3 other repeats of 13/15 base pairs; IaA25R3: *Cryptosporidium hominis* with 25 repeats of TCA and 3 other repeats of 13/15 base pairs; IaA29R3: *Cryptosporidium hominis* with 29 repeats of TCA and 3 other repeats of 13/15 base pairs; IbA13G3: *Cryptosporidium hominis* with 13 repeats of TCA, 3 repeats of TCG; IbA10G2: *Cryptosporidium hominis* with 10 repeats of TCA and 2 repeats of TCG; IeA11G3T3: *Cryptosporidium hominis* with 11 repeats of TCA, 3 repeats of TCG and 3 repeats of TCT

respectively, while the other subtypes occurred in only 1 sample each. Among the *C. parvum* subtypes, on the other hand, IeA10G1 and IICa5G3b were identified in 3 specimens each, while IICa5G3k was identified in 2 specimens (Table 1).

Discussion

Cryptosporidium infections in children and immune-compromised individuals such as HIV-infected persons have been reported from many parts of the world, including southwestern and midwestern Nigeria [4-6,8,10,14-16]. This study was conducted in southeastern Nigeria. The results showed relatively high rates of *Cryptosporidium* infection in diarrheal children and HIV-infected persons in Ebonyi and Nsukka, southeastern Nigeria, with a significantly ($p < 0.05$) higher infection rate in Ebonyi (7.8%) than in Nsukka (3.7%). The difference in *Cryptosporidium* infection rate between Ebonyi and Nsukka is probably due to the fact that residents of Ebonyi have more frequent contact with bodies of water, such as streams and rivers, which are commonly contaminated by sewage and domestic wastewater, as transmission of *Cryptosporidium* spp. is often associated with human and animal fecal contamination of water sources [3,17-20].

Cryptosporidium infections have been associated with low immunity, especially in HIV-infected persons [8,14]. In this study, HIV-infected persons with CD4⁺ cell counts < 200 cells/mm³ had a significantly ($p < 0.05$) higher infection rate (9.7%) than those with CD4⁺ cells > 200 cells/mm³ (5.2%). This finding is consistent with the reports of Ayinmode *et al.* and Adamu *et al.* [5,6] from similar studies conducted in western Nigeria and Ethiopia, respectively. In this study, however, one of the HIV-infected persons, with CD4⁺ cell count of 542 cells/mm³, was infected with *Cryptosporidium*. CD4⁺ cell counts of 500 cells/mm³ are considered as the baseline for immunological competence in humans [21]. The finding in this study, therefore, of *Cryptosporidium* infection in a patient who could be regarded as immune competent, supports previous suggestions by some investigators that *Cryptosporidium* can infect immune-competent individuals [22].

HAART is used to manage HIV infection by reducing viral load, improving immunological status, and reducing opportunistic infections associated with HIV infection [21,23]. In this study, no significant ($p > 0.05$) difference in infection rate was found between HIV-infected persons on HAART and those not on HAART. This finding is consistent with the reports of

Akinbo *et al.* and Ayinmode *et al.* [6,11] that HIV-infected patients on HAART can have *Cryptosporidium* infection.

Review of the clinical records of the HIV-infected persons on HAART co-infected with *Cryptosporidium* in this study revealed that they were placed on a three-drug regimen comprising nucleoside/nucleotide reverse transcriptase inhibitors (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI). None of the patients had HIV-protease inhibitors in their treatment regimen. Of the 8 patients on HAART and co-infected with *Cryptosporidium*, 5 had been on the drugs for over 2 years, 2 for about 1 year and the remaining 1 patient for 5 weeks prior to sampling. The investigation also revealed that the hospitals from which the specimens were collected in this study used HIV-protease inhibitors as the second line of drugs for management of HIV⁺ patients, only in the event of treatment failure. Protease inhibitors have been reported to reduce the occurrence of opportunistic infections in HIV-infected persons [21, 22]. It is therefore possible that *Cryptosporidium* infection of HIV-infected persons on HAART seen in this study could be attributable to the non-inclusion of HIV-protease inhibitors in their treatment regimen. The occurrence of infection in these patients could also be attributable to non-adherence to treatment as is quite common in Nigeria.

A high diversity of *Cryptosporidium* species/subtypes was observed in this study. Although *C. hominis* and *C. parvum* were the dominant *Cryptosporidium* species, one case each of *C. viatorum* and *C. felis* was found. The latter two had been previously seen in HIV-infected patients in Ibadan and Benin City, Nigeria [6,8,11]. Within the two dominant species, 6 *C. hominis* subtypes (IaA27R4, IaA18R3, IaA25R3, IbA10G2, IbA13G3, and IeA11G3T3) in 3 subtype families (Ia, Ib and Ie) and 3 *C. parvum* subtypes (IICa5G3b, IICa5G3k and IIEa10G1) in 2 subtype families (IIC & IIE) were seen in diarrheal children and HIV-infected persons in Ebonyi. Similarly, 2 *C. parvum* subtypes (IICa5G3b and IICa5G3k) and 1 *C. hominis* subtype (IaA29R3) were among the small number of *Cryptosporidium*-positive specimens from HIV-infected persons in Nsukka. The high diversity of *Cryptosporidium* subtypes observed in this study is consistent with previous studies in western Nigeria and other African countries [5,6,8,10,11]. Some of the subtypes seen in this study, such as *C. hominis* subtypes IeA11G3T3, IbA13G3, IbA10G2, and IaA25R3 and *C. parvum* subtypes IICa5G3b and IICa5G3k, have been reported in studies conducted in Benin city, Edo, mid-western Nigeria and Oyo state, southwestern Nigeria

[6,8,10]. Others such as *C. hominis* subtypes IaA18R3, IaA27R4, and IaA29R3 and *C. parvum* subtype IIeA10G1 represent unique subtypes seen in this study.

In this study, a higher occurrence of *C. parvum*, *C. felis*, and *C. viatorum* was seen in suburban areas, whereas *C. hominis* occurred more frequently in the urban area. The differences in *Cryptosporidium* infection rates, species, and subtype distribution between geographic locations or between urban and suburban areas are probably indications of differences in transmission rates in different settings within Nigeria. Nevertheless, anthroponotic transmission appears to be important in cryptosporidiosis epidemiology, as most *Cryptosporidium* infections were caused by the largely human-specific *C. hominis* and the anthroponotic *C. parvum* subtype families IIc and IIe [3]. This is consistent with reports of earlier epidemiological studies in Nigeria and other developing countries. More epidemiological studies are needed to identify reasons for the differences in the transmission of *Cryptosporidium* species and subtypes seen in this study among patient populations and geographic areas.

Conclusions

The high diversity of *Cryptosporidium* species and subtypes observed in this study (including unique *Cryptosporidium hominis* possibly lacking the gp60 gene [unsubtyped], *C. hominis* subtypes IaA18R3, IaA27R4, and IaA29R3, and *C. parvum* subtype IIeA10G1) is indicative of the need for consistent surveillance for zoonotic parasite in the region. The study further revealed that *Cryptosporidium* transmission in study area is probably anthroponotic in nature. Hence, further studies are needed to identify the exact route(s) of *Cryptosporidium* transmission in the studied areas, which will aid in controlling of the spread of the infectious agent.

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