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Molecular epidemiology of *Cryptosporidium* in HIV/AIDS patients in Malaysia

Asma Iqbal[1,2], Sim, B.L.H.[3], Brent R. Dixon[2], Johari Surin[1] and Yvonne AL Lim[1*]
[1]Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
[2]Microbiology Research Division, Bureau of Microbial Hazards, Food Directorate, Health Canada, Banting Research Centre, 251 Sir Frederick Banting Driveway, P.L.2204E, Ottawa, Ontario, K1A 0K9 Canada
[3]Infectious Disease Unit, Department of Medicine, Hospital Sungai Buloh, 47000 Sungai Buloh, Selangor Darul Ehsan, Malaysia
[*]Corresponding author email: limailian@um.edu.my
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Abstract. Cryptosporidiosis is a particular concern in immunocompromised individuals where symptoms may be severe. The aim of this study was to examine the epidemiological and molecular characteristics of *Cryptosporidium* infections in HIV/AIDS patients in Malaysia in order to identify risk factors and facilitate control measures. A modified Ziehl-Neelsen acid fast staining method was used to test for the presence of *Cryptosporidium* oocysts in the stools of 346 HIV/AIDS patients in Malaysia. Standard coproscopical methods were used to identify infections with other protozoan or helminths parasites. To identify the species of *Cryptosporidium*, DNA was extracted and nested-PCR was used to amplify a portion of the SSU rRNA gene. A total of 43 (12.4%) HIV-infected patients were found to be infected with *Cryptosporidium* spp. Of the 43 *Cryptosporidium*-positive HIV patients, 10 (23.3%) also harboured other protozoa, and 15 (34.9%) had both protozoa and helminths. The highest rates of cryptosporidiosis were found in adult males of Malay background, intravenous drug users, and those with low CD4 T cell counts (i.e., < 200 cells/mm^3). Most were asymptomatic and had concurrent opportunistic infections mainly with *Mycobacterium tuberculosis*. DNA sequence analysis of 32 *Cryptosporidium* isolates identified *C. parvum* (84.3%), *C. hominis* (6.3%), *C. meleagridis* (6.3%), and *C. felis* (3.1%). The results of the present study revealed a high prevalence of *Cryptosporidium* infection in hospitalized HIV/AIDS patients. The results also confirmed the potential significance of zoonotic transmission of *C. parvum* in HIV-infected patients, as it was the predominant species found in this study. However, these patients were found to be susceptible to a wide range of *Cryptosporidium* species. Epidemiological and molecular characterization of *Cryptosporidium* isolates provides clinicians and researchers with further information regarding the origin of the infection, and may enhance treatment and control strategies.

INTRODUCTION

Infection with the protozoan parasite *Cryptosporidium* is a major public health concern as the ingestion of low numbers of oocysts can cause severe diarrheal disease (cryptosporidiosis) (Nuchjangreed *et al.*, 2008). *Cryptosporidium* species infect humans and many other vertebrate animals. In humans, healthy individuals may clear the infection in less than a month, but those whose immune systems are compromised, particularly AIDS patients, transplant patients, and cancer patients, suffer prolonged and potentially fatal episodes of diarrhea (Nuchjangreed *et al.*, 2008). Diarrhea is a common complication of HIV infections, inducing weight loss and cachexia and occurring in almost 90% of AIDS patients in developing countries (Adesiji *et al.*, 2007). *Cryptosporidium* is a well-established cause of diarrhea among HIV infected patients worldwide, with prevalence of infection ranging from 3% in developed countries to

50% in developing countries (O'Connora *et al.*, 2011). Transmission is often through the fecal-oral route, via, person-to-person spread, zoonotic transmission from animals, or possibly airborne contact (Leoni *et al.*, 2006). Waterborne cryptosporidiosis is associated with drinking water sources, or recreational water, contaminated with either human or animal feces (Fayer *et al.*, 2000). Fecal contamination of drinking water has resulted in major waterborne outbreaks of cryptosporidiosis (Karanis *et al.*, 2007). Additionally, *Cryptosporidium* is now increasingly considered an important foodborne pathogen (Smith *et al.*, 2007) causing a disease of socioeconomic significance worldwide. Cryptosporidiosis has been associated with the consumption of a variety of foods, particularly fresh produce. *Cryptosporidium parvum* oocysts have been detected in fresh produce such as green leafy vegetables and have resulted in numerous foodborne outbreaks (Dixon *et al.*, 2013).

Cryptosporidium infections in HIV-infected individuals can reduce both quality and duration of life, especially in those who are severely immunosuppressed with CD4 T cell counts of < 200 cells/mm^3 (Sadraei *et al.*, 2005). Low CD4 cell count has been significantly associated with diarrhea caused by *Cryptosporidium* infections in HIV-infected patients (Sadraei *et al.*, 2005). Generally, with increased CD4 T cell levels, spontaneous clearing of the parasite takes place (Gupta *et al.*, 2008), and chronic diarrhea and cryptosporidial infection often resolves. *Cryptosporidium* infection alone has been associated with low CD4 T cell counts (Brink *et al.*, 2000). Several intestinal parasitic pathogens which have been reported in HIV patients include *Cryptosporidium parvum*, *Cystoisospora belli*, microsporidia *(Enterocytozoon bieneusi*, *Encephalitozoon intestinalis)*, *Giardia duodenalis*, *Entamoeba histolytica/ dispar*, *Cyclospora cayetanensis*, *Ascaris lumbricoides*, *Trichuris trichiura*, hookworms and *Strongyloides stercoralis* (Ramakrishnan *et al.*, 2007; Gupta *et al.*, 2008, Asma *et al.*, 2012). With impaired immunity especially in patients with low immune level (CD4 counts < 200 cells/mm^3), infections with intestinal parasites may result in diarrheal symptoms (Daryani *et al.*, 2009). With the introduction of highly active antiretroviral therapy (HAART) which partially restores the immune function, the incidence of opportunistic parasite infection such as cryptosporidiosis has declined (Hung *et al.*, 2007).

Currently, there are more than 27 recognized species of *Cryptosporidium* infecting a wide variety of animals including humans (Fayer *et al.*, 2010; Traversa, 2010; Ren *et al.*, 2012). Of these, at least seven have been found to infect HIV-infected individuals. Due to the weakened immunological status of immunocompromised individuals, infections with *Cryptosporidium* are not only caused by the predominant human species (i.e., *C. hominis* and *C. parvum*) but these individuals are also susceptible to infections by other minor human species, especially *C. meleagridis*, *C. felis*, *C. muris*, *C. canis* and *C. suis*. The distribution of *Cryptosporidium* species varies from one country to another and from one region to another (Xiao, 2010). The use of molecular methods has improved recognition of the diversity of species that infect humans and animals, and has facilitated epidemiological studies on these species. A variety of PCR-based techniques have been used for genetic characterization of *Cryptosporidium*, and a number of genetic loci have been identified as targets for the detection of species as well as for genotype identification of different *Cryptosporidium* isolates (Nichols *et al.*, 2003; Xiao *et al.*, 2004). Studies of the small subunit (SSU) rRNA gene have shown that the ability to amplify this gene fragment from different species and genotypes of the organism with one set of primers makes this locus a gold standard, and the most appropriate target for screening, where the species and genotypes of *Cryptosporidium* are unknown (Xiao *et al.*, 2004).

In Malaysia, the first cryptosporidiosis case was reported in 1984 (Che Ghani *et al.*, 1984). So far, studies in Malaysia have only focused on determining the prevalence rates of cryptosporidiosis in HIV/AIDS patients (Kamel *et al.*, 1994; Lim *et al.*, 2005

& 2011; Zaidah *et al*., 2008; Asma *et al*., 2011). Molecular tools are very useful in tracking infection and contamination sources. Lim *et al*. (2011) published the first report of *C. hominis*, *C. meleagridis* and *C. felis* from Malaysian HIV patients. The sequencing of amplicons derived from SSU rRNA revealed that *C. parvum* was the most commonly detected species followed by *C. hominis*, *C. meleagridis* and *C. felis*. Sequencing of the 60-kDa glycoprotein (gp60) gene identified *C. parvum* subgenotype IId and *C. hominis* subgenotypes Ia, Ib, Id, Ie and If in HIV patients (Lim *et al*., 2011). Moreover, another study targeting the gp60 gene identified *C. parvum* IIa, IId and *C. hominis* Ia and Ib subgenotypes in Malaysian HIV patients (Iqbal *et al*., 2012). There is little information on the distribution of *Cryptosporidium* species in Malaysia, and the susceptibilities of HIV/AIDS individuals to genotypes of *C. parvum*, and to other species of *Cryptosporidium*, have not been extensively studied. Therefore, the current study was undertaken to detect the genotypes and species of *Cryptosporidium* in fecal samples of HIV/AIDS individuals in order to determine their public health significance. The results of these investigations will provide public health information for HIV-infected individuals who are vulnerable to a wide range of *Cryptosporidium* species and subtypes. The knowledge of the prevalence and characterization of *Cryptosporidium* species is important in terms of epidemiological ramifications; and would facilitate effective and control strategy programs intended to reduce the impact of cryptosporidiosis amongst HIV patients.

MATERIALS AND METHODS

The study was conducted from March 2008 to June 2010 on 346 HIV-infected individuals from three different hospitals in Malaysia, namely: Hospital Sungai Buloh, Selangor; University Malaya Medical Centre, Kuala Lumpur; and Hospital Raja Zainab Perempuan II, Kelantan. Ethical clearance and patient's consent according to the institutional ethical guidelines (IRB Ref. No. 655.17, MOH-NMRR ID: # 09-286-3930) were obtained prior to the commencement of the study.

For those patients who have provided fecal samples the following information was collected: socio-demographic characteristics (age, gender, ethnic background and mode of transmission), clinical and immunological data and HIV treatment regime/antiretroviral therapy commonly administered i.e., nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI) and protease inhibitors (PI). This information was obtained from patient's medical records with their consent and the permission of health authorities. Diarrhea was defined based on two criteria: a) macroscopic examination (i.e., watery or loose stools), b) clinically characterized diarrhea (i.e., transient diarrhea, persistent diarrhea and bloody diarrhea). Patients were considered to have diarrhea if there were $\geq$3 loose or liquid stools in a 24 hour period. A diarrheal episode was considered to end when the participant had $\geq$7 consecutive days without diarrhea.

Single stool samples from all 346 hospitalized patients were collected in sterile screw-capped fecal containers with 2.5% potassium dichromate solution as a preservative (Asma *et al.*, 2011), transported to the Department of Parasitology, University of Malaya and stored at 4°C prior to analysis. Epidemiological data were analyzed using the SPSS program for Windows version 17 (SPSS Inc., Chicago, IL, USA) for data entry and statistical analysis. Descriptive statistics were mainly used to describe the characteristics of the study population. Qualitative data were determined and presented as frequencies and percentages. Statistical analysis was evaluated by Chi-Square test with significance value of $p < 0.05$ used for all tests.

Stool samples were subjected to coproscopic examination. Small portions (pea sized) of fecal samples were mixed with a drop of iodine on a microscopic slide and covered with a cover slip. Slides were then examined under 100X and 400X magnifications to detect cysts, oocysts, ova and larvae of intestinal parasites such as *Entamoeba histolytica/dispar*, *Giardia*

duodenalis, *Ascaris lumbricoides* and *Trichuris trichiura*. Modified Ziehl-Neelsen acid fast stain was used for the microscopic identification of *Cryptosporidium* at 400X. *Cryptosporidium* oocysts appeared as bright rose-pink spheres (5 ±1µm) on a pale green background.

Microscopically-positive *Cryptosporidium* samples were used for molecular characterization. Concentrated oocysts from fecal samples were acquired by mixing a small portion of the feces with 10 ml of distilled water and sieving through cotton gauze. The suspension was then centrifuged at 1,500 x g for 10 min and the supernatant was discarded. The pellet was resuspended in 5 ml of distilled water and subjected to immunomagnetic separation (IMS). IMS was carried out using Dynabeads® GC-Combo kit (Dynal, cat. No. 730.02, Oslo, Norway) according to the manufacturer's instructions.

DNA was extracted from the IMS-isolated oocysts using QIAamp DNA Mini kit (QIAGEN, cat. No. 51306, Germany), using a slightly modified protocol as described in Iqbal *et al*. (2012). Nested-PCR was performed to amplify a partial polymorphic region of SSU rRNA gene, according to Nichols *et al.* (2003). Positive (extracted DNA of *C. parvum* oocysts purchased from Waterborne, Inc., New Orleans, LA, USA) and negative (DNase free water instead of DNA template) controls, were included in each round of amplification. The PCR product was analyzed by electrophoresis in a 2% agarose gel and visualization of SYBR® Safe (Molecular Probes, Inc. Eugene, OR, USA) stained DNA was performed by ultraviolet light illumination of gels using a UV transilluminator.

Following electrophoresis, DNA was purified using QIAquick PCR purification kit (QIAgen, cat. No. 28104, Germany), according to the manufacturer's protocol. DNA sequencing was carried out by Medigene (Solgent Co. Ltd, South Korea). For *Cryptosporidium* (SSU rRNA), DNA sequencing of the secondary PCR product was done in both directions. Sequences obtained were compared using the basic local alignment search tool (BLAST; www.ncbi.nlm.nih.gov/blast) with those available in current gene databases and published in peer-reviewed international scientific journals. SSU rRNA sequences were compared with reference sequences of *C. parvum* (GenBank accession no. AB513881), *C. hominis* (GenBank accession no. DQ286403), *C. meleagridis* (GenBank accession no. AF112574) and *C. felis* (GenBank accession no. AF112575).

RESULTS

Microscopic examination of all 346 fecal samples obtained from HIV/AIDS patients indicated that the total number of *Cryptosporidium*-positive samples was 43 (12.4%). The detailed demographic features of these 43 *Cryptosporidium*-positive HIV patients included 4 (9.3%) children (age range: 1 - 12 years; mean age: 2.3 years), and 39 (90.7%) adults (age range: 22 to 54 years; mean age: 35.5 years). Thirty-nine (90.7%) were males and 4 (9.3%) were females. The *Cryptosporidium*-positive individuals were of various ethnic backgrounds, including 24 (55.8%) Malays, 10 (23.3%) Chinese, 4 (9.3%) Indians and 5 (11.6%) foreigners (i.e., Myanmarese). Four (9.3%) patients were symptomatic (i.e., had watery diarrhea) whilst 39 (90.7%) were asymptomatic. One *Cryptosporidium*-positive patient died following the study due to a co-infection with *Mycobacterium tuberculosis*. As some patients did not give consent for their clinical records to be accessed, clinical information such as mode of HIV/AIDS transmission, CD4 T cell counts, use of highly active anti-retroviral therapy (HAART), and the presence of opportunistic infections was only obtained for 34 of the 43 *Cryptosporidium*-positive HIV-infected individuals. Of these 34 *Cryptosporidium*-positive HIV patients, 20 (58.8%) were intravenous drug users (IVDU), 7 (20.6%) were heterosexuals, and 7 (20.6%) were patients who chose not to disclose their mode of transmission (denoted as "unknown"). No homosexual patients were found to have *Cryptosporidium* infection in the current study. No demographic variables

were found to be significantly associated with *Cryptosporidium* infection in the studied population.

Of the 43 cases, CD4 T cell count data was available for only 34 *Cryptosporidium*-positive HIV patients (those who gave consent for their clinical records). The majority, 27 (79.4% of 34) patients, had CD4 counts of < 200 cells/mm_3, 6 (17.6%) having CD4 counts > 200 cells/mm^3, whilst 1 (2.9%) patient did not have any record on CD4 count. The frequencies of T cell counts were further divided into various categories according to Kurniawan *et al.* (2009) as follows: a) 18 (52.9%) patients having CD4 counts of ≤ 50 cells/mm^3; b) 3 (8.8%) had 51-100 cells/mm^3; c) 6 (17.6%) had 101-200 cells/mm^3; d) 4 patients (11.8%) had CD4 counts 201-400 cells/mm^3 and e) 2 (5.9%) had CD4 counts > 400 cells/mm^3.

At the time of sample collection, there were 19 (55.9% of 34) patients who were on HAART and 15 (44.1%) who were not on HAART due to reasons such as side effects, non-compliance and late presentation. Generally, the most common combinations of NRTI and NNRTI in those infected with *Cryptosporidium* were [d4T (Stavudine) and 3TC (Lamivudine)] with Storcin (Efavirenz) (42.1%, 8 of 19) and d4T and 3TC with Viramune (Nevirapine) (31.5%, 6 of 19). One *Cryptosporidium* positive patient was on PI therapy (i.e., Indinavir).

In addition, 22 (64.7%) of the 34 *Cryptosporidium*-positive HIV patients also had co-infection with opportunistic infections (OIs). The most commonly identified opportunistic infections recorded in these 22 HIV/AIDS patients were with *Mycobacterium tuberculosis* (11, 50.0% of 22), followed by cerebral toxoplasmosis (5, 22.7%), disseminated or extrapulmonary *Mycobacterium tuberculosis* infection, candidiasis, cryptococcosis (3 each, 13.6%), *Salmonella* septicaemia and *Pneumocystic carinii* pneumonia (2 each, 9.1%).

Of the 43 *Cryptosporidium*-positive patients, 10 (23.3%) also harboured other protozoans and 15 (34.9%) had both protozoans and helminths (Table 1). The most common type of protozoa detected in *Cryptosporidium*-positive HIV patients was *Entamoeba histolytica/dispar* (41.9% of 43), followed by *Cystoisospora belli* (18.6%), *Giardia duodenalis* (14.1%) and *Cyclospora cayetanensis* (4.7%). Helminths observed included *Ascaris lumbricoides* (25.6%), *Trichuris trichiura* (9.3%) and hookworm (2.3%) (Table 1). The combination of mixed infections of *Cryptosporidium* with *E.*

Table 1. Prevalence of intestinal parasites detected in *Cryptosporidium*-positive HIV/AIDS patients (n = 43)

Parasites	No. infected	%
Protozoa		
Entamoeba histolytica/dispar	18	41.9
Cystoisospora belli	8	18.6
Giardia duodenalis	6	14.1
Cyclospora cayetanensis	2	4.7
Helminths		
Ascaris lumbricoides	11	25.6
Trichuris trichiura	4	9.3
Hookworm	1	2.3
Intestinal parasitic infections IPIs		
Cryptosporidium infection (Single infection)	18	41.9
Cryptosporidium + Protozoa infections	10	23.3
Cryptosporidium + Protozoa + Helminths infections	15	34.9

histolytica/dispar and *A. lumbricoides* was the most widespread among the HIV/AIDS patients.

All microscopically *Cryptosporidium*-positive samples (i.e., 43 samples) were genetically analyzed using the nested-PCR protocol and produced a 435bp fragment which has been reported to be valid in differentiating all reported *Cryptosporidium* species and genotypes. By utilizing nested-PCR targeting the SSU rRNA gene, amplicons were obtained from 36 (83.7% of 43) *Cryptosporidium*-positive HIV patients. Four of these 36 PCR-positive *Cryptosporidium* samples did not show good DNA sequences. No PCR amplicon was detected in any of the microscopically negative specimens.

In order to determine the species of *Cryptosporidium* isolates from the HIV/AIDS patients, a total of 32 isolates were successfully sequenced in both directions. BLAST results of the 32 sequences showed that HIV patients harboured four different *Cryptosporidium* species i.e., 27 (84.3%) were identified as *C. parvum*, 2 (6.3%) *C. hominis*, 2 (6.3 %) *C. meleagridis* and 1 (3.1%) *C. felis* (Table 2). The 32 nucleotide sequences of the SSU rRNA gene of *Cryptosporidium* isolates from the present study were deposited in GenBank under accession numbers HQ450658 to HQ450673, HQ450675, HQ450677 to HQ450681, HQ450683, HQ450685 to HQ450690, and HQ729707 to HQ729709, representing isolates of *C. parvum*, *C. hominis*, *C. meleagridis* and *C. felis* respectively.

Demographic and clinical characteristics of HIV/AIDS patients according to *Cryptosporidium* species indicated that the majority of the 27 HIV/AIDS patients infected with *Cryptosporidium parvum* were adults (92.5%, 25 of 27), 24 (88.8%) were male and 3 (11.1%) were females. Most of them were Malay (37.0%, 10), IVDU (63.0%, 17), having CD4 count < 50 cells/mm^3 (55.5%, 15), undergoing HAART (55.5%, 15), and had concurrent OIs (66.6%, 18). There were 11 (40.7%) *C. parvum* infected patients who had co-infections with *Mycobacterium tuberculosis*. There were two patients (7.4%) who were symptomatic (i.e., had diarrhea) and one of the symptomatic patients died during the study period. Generally, those who were infected with *C. parvum* also had co-infections with a variety of other intestinal parasites such as *E. histolytica/dispar* (55.5%, 15 of 27), *C. belli* and *A. lumbricoides* (29.6%, 8 each), *G. duodenalis* (18.5%, 5), *T. trichiura* (11.1%, 3), *C. cayetanensis* and hookworm (3.7%, 1 each) (Table 2).

The two patients infected with *Cryptosporidium hominis* were both male, one of them was a Malay IVDU and the other a Chinese heterosexual. Both had CD4 counts < 50 cells/mm^3 and were undergoing HAART. The *C. hominis* positive Chinese patient had co-infections with *G. duodenalis* and *A. lumbricoides* (Table 2). This patient was also co-infected with OIs, mainly Herpes simplex and Kaposi's sarcoma (Table 2).

The two patients infected with *Cryptosporidium meleagridis* were Malay adult males. One patient was a heterosexual with a CD4 count between 51-100 cells/mm^3 and was not on HAART. This patient also had cryptococcosis as well as infection with *E. histolytica/dispar* (Table 2). The other was an IVDU, with a CD4 count of 101-200 cells/mm^3 and was on HAART regimen.

The only patient infected with *Cryptosporidium felis* was Malay male; who did not disclose his mode of HIV transmission. This patient had a CD4 count of < 50 cells/mm^3 and was not on HAART therapy. He also had cerebral toxoplasmosis and *A. lumbricoides* infection (Table 2).

DISCUSSION

In this study, we found a high prevalence (12.4%) of *Cryptosporidium* oocysts in fecal samples from HIV/AIDS patients in Malaysia. These results are in accordance with previous studies carried out among HIV-positive IVDU patients in Malaysia (Kamel *et al*., 1994), and in hospitalized HIV/AIDS patients in Kota Bharu (Zaidah *et al*., 2008). Another Malaysian study reported a much lower prevalence (3%) of cryptosporidiosis among HIV patients (Lim *et al*., 2005). The prevalence of cryptosporidiosis in HIV patients varies among studies, depending on where they were conducted, age of the

Table 2. Demographic, clinical characterization and species distribution of *Cryptosporidium* in HIV/AIDS patients (n = 32)

	C. parvum n = 27 (%)	*C. hominis* n = 2 (%)	*C. meleagridis* n = 2 (%)	*C. felis* n = 1 (%)	OR (95% CI)	p
Age (years)						
Children (≤ 12)	2 (7.4)	–	–	–		
Adults (22 to 54)	25 (92.5)	2 (100.0)	2 (100.0)	1 (100.0)	0.34 (0.04-2.78)	0.308
Gender						
Male	24 (88.8)	2 (100.0)	2 (100.0)	1 (100.0)	1.17 (0.11-12.58)	0.692
Female	3 (11.1)	–	–	–		
Ethnic Groups						
Malay	10 (37.0)	1 (50.0)	2 (100.0)	1 (100.0)	6.07 (1.13-32.41)	0.205
Chinese	9 (33.3)	1 (50.0)	–	–	0.22 (0.02-1.98)	0.237
Indians	4 (14.8)	–	–	–	0.61 (0.06-6.12)	0.569
Foreigners	4 (14.8)	–	–	–	0.61 (0.06-6.12)	0.569
Mode of Transmission						
Heterosexual	3 (11.1)	1 (50.0)	1 (50.0)	–	10.22 (1.49-69.76)	0.203
IVDU	17 (63.0)	1 (50.0)	1 (50.0)	–	0.39 (0.07-2.17)	0.393
Unknown	6 (22.2)	–	–	1 (100.0)		
NA	1 (3.7)	–	–	–		
[a]CD4 counts/ mm^3						
0-50	15 (55.5)	2 (100.0)	–	1 (100.0)		
51-100	2 (7.4)	–	1 (50.0)	–		
101-200	4 (14.8)	–	–	–		
201-400	4 (14.8)	–	–	–		
> 400	1 (3.7)	–	–	–		
NA	1 (3.7)	–	–	–		
Intestinal parasites						
Protozoa						
Entamoeba histolytica/dispar	15 (55.5)	–	1 (50.0)	–	2.81 (0.63-12.41)	0.191
Cystoisospora belli	8 (29.6)	–	–	–	0.74 (0.60-0.91)	0.082
Giardia duodenalis	5 (18.5)	1 (50.0)	–	–	2.11 (0.22-20.26)	0.455
Cyclospora cayetanensis	1 (3.7)	–	–	–	0.36 (0.02-6.38)	0.485
Helminths						
Ascaris lumbricoides	8 (29.6)	1 (50.0)	–	1 (50.0)	1.04 (0.22-4.84)	0.640
Trichuris trichiura	3 (11.1)	–	–	–	1.17 (0.11-12.58)	0.692
Hookworm	1 (3.7)	–	–	–	0.96 (0.90-1.03)	0.721
Opportunistic infections						
Yes	18 (66.6)	1 (50.0)	1 (50.0)	0	0.71 (0.15-3.25)	0.485
No	8 (29.6)	1 (50.0)	1 (50.0)	1 (100.0)		
NA	1 (3.7)	–	–	–		
Mycobacterium tuberculosis	11 (40.7)	–	–	–	0.57 (0.41-0.80)	0.034
Mycobacterium: disseminated or extrapulmonary	3 (11.1)	–	–	–	0.90 (0.80-1.01)	0.751
Salmonella septicemia	2 (7.4)	–	–	–	0.92 (0.82-1.03)	0.579
Candidiasis	2 (7.4)	–	–	–	0.92 (0.82-1.03)	0.579
Pneumocystis carinii pneumonia	2 (7.4)	–	–	–	0.92 (0.82-1.03)	0.579
Cryptococcosis	2 (7.4)	–	1 (50.0)	–	0.58 (0.04-7.42)	0.566
Histoplasmosis	1 (3.7)	–	–	–	0.96 (0.89-1.03)	0.765
Herpes simplex	–	1 (50)	–	–	1.14 (0.88-1.48)	0.235
Cerebral Toxoplasmosis	4 (14.8)	–	–	1 (100)	1.27 (0.12-13.35)	0.666
Primary lymphoma of brain	1 (3.7)	–	–	–	0.96 (0.89-1.03)	0.765
Kaposi's sarcoma	–	1 (50)	–	–	1.14 (0.88-1.48)	0.235
Wasting syndrome	1 (3.7)	–	–	–	0.96 (0.89-1.03)	0.765
HIV-related encephalopathy	1 (3.7)	–	–	–	0.96 (0.89-1.03)	0.765

[a]: No statistics are computed because CD4 range is a constant
NA: Not Available.

patients, the stage of disease (HIV/AIDS) and the laboratory methods used (Chhin *et al*., 2006; Aseefa *et al*., 2009). Results of the present study are in concordance with the studies carried out in other parts of the world such as Iran (Meamar *et al*., 2007; Daryani *et al*., 2009), Brazil (Botero *et al*., 2003), India (Ramakrishnan *et al*., 2007; Gupta *et al*., 2008;), Thailand (Nuchjangreed *et al*., 2008; Srisuphanunt *et al*., 2008); Cambodia (Chhin *et al*., 2006); Ethiopia (Aseefa *et al*., 2009) and Nigeria (Adesiji *et al*., 2008).

The occurrence of *Cryptosporidium* in both symptomatic and asymptomatic cases indicated a high risk of infection of this parasite (Srisuphanunt *et al*., 2008). The present study revealed predominantly asymptomatic HIV carriers of *Cryptosporidium*. Ten *Cryptosporidium*-infected HIV patients (23.3% of 43) harboured other protozoan parasites and 15 (34.9%) were positive for both protozoa and helminths, whereas no *Cryptosporidium*-positive patients had co-infections strictly with helminths. The most common multi-parasitic co-infections observed among these patients were with *E. histolytica/dispar* and *A. lumbricoides*. Our results are in agreement with the findings of Adesiji *et al.* (2007), which detected a high prevalence of *T. trichiura*, *A. lumbricoides*, and *G. duodenalis* in Nigerian *Cryptosporidium*-positive HIV patients.

Our results indicate that adult patients harbour greater diversity of other intestinal parasites as compared to children (those 12 years and below). Age related increase in intestinal parasites prevalence has been previously reported in adults (Assefa *et al*., 2009). However, there is limited data of intestinal parasitic infection available in HIV/AIDS patients of 12 years of age and below whether within Malaysia or globally (Tumwine *et al*., 2005; Ajjampur *et al*., 2008). It was interesting to note that children who were 12 years of age and below only had single infections of *Cryptosporidium*. None harboured any other intestinal parasites, whereas in other studies, children were found to be infected with other intestinal parasites as well (Menon *et al*., 2001; Certad *et al*., 2005; Asma *et al*., 2011). The variation in the prevalence of parasitic infection in current findings and reports from other countries could be due to differences in the study population, the stage of disease, the laboratory methods used and the fact that only single stool specimens were examined in the present study. Geographic, socio-economic and ethnic differences could explain the variation of our findings with other developing countries.

Current findings showed that there was no significant difference in the prevalence of parasitic infections between males and females ($p > 0.05$). In male *Cryptosporidium*-positive HIV patients, protozoa infections were more common compared to helminths. However in female *Cryptosporidium*-positive HIV patients, only single protozoa infections (25%, 1 of 4) were found; neither helminths nor any mixed infections of protozoa and helminths were detected. Zali *et al.* (2004), also noted no significant difference between males and females ($p > 0.05$). Data analysis indicated that for mode of HIV transmission in *Cryptosporidium*-positive patients, IVDU and heterosexual patients were infected with all types of other parasites except hookworms (for IVDU) and *Cyclospora cayetanensis* (for heterosexual). To the best of our knowledge, none of the available reports stated an association with other intestinal parasites with the mode of transmission.

Detailed analysis based on different categories of CD4 T cell counts highlighted that *Cryptosporidium*-positive patients with CD4 counts of $\leq$ 50 cells/mm^3 were infected with many other types of parasites. Researchers from Iran and Indonesia determined the same pattern of parasitic infection in different categories of CD4 cell counts, and it was reported that *Cryptosporidium*-positive HIV patients were significantly associated with chronic diarrhea especially among those with CD4 count of $\leq$ 50 cells/mm^3 (Daryani *et al*., 2009; Kurniawan *et al*., 2009). This may be due to immunocompromised patients being more susceptible to parasitic infections or being unable to clear infection once established.

The clinical profile of *Cryptosporidium*-positive HIV patients included a wide range of other opportunistic infections including *Mycobacterium tuberculosis*, cerebral toxoplasmosis, candidiasis, cryptococcosis, *Salmonella* septicaemia and *Pneumocystis carinii* pneumonia. In India, tuberculosis is the most common opportunistic infection in HIV/AIDS patients (Kumarasamy *et al*., 2005). In Nepal, 34.8% of 23 *Cryptosporidium*-positive HIV patients also had toxoplasmosis (7.9%), *Pneumocystis carinii* pneumonia (15.3%), cryptococcal meningitis and cryptococcemia (7.6%), and herpes simplex (7.6%) (Ghimire *et al*., 2004). Based on these findings, the only parameter unequivocally related to opportunistic infections in HIV/AIDS patients is the immune status. HAART regimen may help to reduce acquisitions of these opportunistic infections in immuno-compromised patients.

The most common PCR target gene utilised in molecular studies is SSU rRNA. Lim *et al.* (2011), published the first report of *C. hominis*, *C. meleagridis* and *C. felis* from Malaysian HIV patients. The sequencing of amplicons derived from SSU rRNA revealed that *C. parvum* was the most commonly detected species (64%, 16 isolates of 25 cases). Another study in Malaysia reported only *C. parvum* in 9 HIV patients (Zaidah *et al*., 2008), with none of the other species encountered. Previously we published the distribution of cryptosporidial species and subgenotypes in HIV-infected individuals in Malaysia (Iqbal *et al*., 2012). Subgenotype analysis of gp60 gene identified 18 isolates as *Cryptosporidium*-positive, with 72.2% of the 18 identified as *C. parvum* and 27.7% as *C. hominis*. Gp60 analysis revealed *C. parvum* belonging to subgenotypes IIa and IId whereas; *C. hominis* was represented by subgenotypes Ia and Ib. The predominance of the *C. parvum* subgenotypes signified the possibility of zoonotic as well as anthroponotic transmission of cryptos-poridiosis in these individuals (Iqbal *et al*., 2012).

HIV/AIDS patients are known to be susceptible to a broad spectrum of species and genotypes of *Cryptosporidium* (Xiao *et al*., 2001; Cama *et al*., 2007). Among the zoonotic species detected in the present study, a high proportion of isolates (84.3%) were *C. parvum*, while *C. meleagridis* and *C. felis* were also identified in some patients. It was noted that infection rates were higher for *C. parvum* compared to *C. hominis* in HIV patients in this study. This situation was also observed in Iran where *C. parvum* was found to be more common than *C. hominis* in HIV-infected adults, whereas no significant difference in the distribution of *Cryptos-poridium* species (i.e., *C. parvum* versus *C. hominis*) was observed in children (Meamar *et al*., 2007). Transmission routes for the rare *Cryptosporidium* species are unclear. As HIV/AIDS patients are more susceptible to a wider host range, it is possible that *C. meleagridis*-infected patients acquired their *Cryptosporidium* infections via contact with aviary birds (Essid *et al*., 2008). Studies have shown that HIV/AIDS patients have acquired *C. felis* infections from their pets, especially cats (Matos *et al*., 2004). However, in the present study, no data on animal contact were available from HIV/AIDS patients; therefore concrete inference could not be made. It is obvious based on these studies that the distribution of the infecting species of *Cryptosporidium* varies according to the ethnic background, age of subjects, and level of immune status, season, and the geographic location.

Given that the presence of infection due to zoonotic *Cryptosporidium* species in HIV/AIDS patients is significant, it is crucial that immunocompromised patients are being informed on the potential of acquiring cryptosporidiosis from infected animals and contaminated water. The high proportion of zoonotic species in the present study suggests that a comprehensive molecular epidemiological study is required to evaluate the actual significance of zoonotic transmission in communities living in close contact with animals (i.e., farm animals, companion animals, or wildlife). This information is vital for assessing clinical and epidemiologic implications and can be utilized as an important tool for public health measures against cryptosporidiosis in

Malaysia. Crucially, in order to develop effective disease reduction strategies related to *Cryptosporidium* and parasitic infections, a smart partnership should be built among Ministry of Health, Malaysia, especially the physicians caring for HIV patients, non-governmental agencies affiliated with these individuals such as Malaysian AIDS Council, as well as the targeted HIV/AIDS patients themselves. Granted that the transmission of enteric parasites, such as *Cryptosporidium* spp., is commonly associated with poor personal hygiene, effective preventive and control measures should take into account promotion of proper hand washing practices, especially after using the toilet or handling animals, and before preparing food. Hence, there is an urgent need to implement an innovative and integrated control program to significantly reduce the prevalence and intensity of *Cryptosporidium* spp. infections among HIV-infected individuals. The present study highlights the diversity of *Cryptosporidium* infections in humans in Malaysia and the risk factors for infection in HIV patients. It is anticipated that information of this type will be of great value in developing such a program, as well as in informing risk assessments and developing policies and guidelines related to the control of cryptosporidiosis in Malaysia and elsewhere.

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