Profile of the molecular epidemiological analysis based on the SSU rRNA gene of

_Cryptosporidium_ in patients infected with HIV/AIDS in Malaysia

Asma Iqbal¹,², Benedict LH Sim³, Brent R. Dixon², Johari Surin¹, Yvonne AL Lim¹,*

¹Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
²Microbiology 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
³Infectious Disease Unit, Department of Medicine, Hospital Sungai Buloh, 47000 Sungai Buloh, Selangor Darul Ehsan, Malaysia
Abstract

**Background:** Infection with the protozoan parasite *Cryptosporidium* is an important public health concern worldwide. Cryptosporidiosis is of particular concern in immunocompromised individuals where symptoms may be severe. The aim of this study therefore, 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.

**Methods:** A modified Ziehl-Neelsen acid fast staining method was used to test for the presence of *Cryptosporidium* spp. oocysts in the stools of 346 HIV-infected patients in Malaysia. Standard coproscopical methods were used to identify infections with other protozoan or helminth parasites. Clinical and demographic information was accessed wherever possible for these patients. To identify the species of *Cryptosporidium*, oocysts were concentrated from stool samples by immunomagnetic separation, DNA was extracted, and nested-PCR was used to amplify a portion of the SSU rRNA gene. DNA sequence analysis was then performed on the PCR products.

**Results:** 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 cells (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%).
**Conclusions:** The results of the present study revealed a high prevalence of *Cryptosporidium* infection in hospitalized HIV/AIDS patients. Interestingly, most of these patients were found to be asymptomatic. The results of this study 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.

**Keywords:** *Cryptosporidium*, molecular characterization, epidemiology, SSU rRNA, HIV/AIDS

**Background**

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) [1]. *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 [1]. Transmission is often through the fecal-oral route, via, person-to-person spread, zoonotic transmission from animals, person-to-person spread, or possibly airborne contact [2]. Waterborne cryptosporidiosis is associated with drinking water sources contaminated with either human or animal feces [3]. Cryptosporidiosis has also been associated with the consumption of certain foods, particularly fresh produce. *C. parvum* cysts have been detected in raw vegetables and green leafy vegetables and have resulted in numerous foodborne outbreaks [4,5].
*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$^3$ [6]. Low CD4 cell count has been significantly associated with diarrhea caused by *Cryptosporidium* infections in HIV-infected patients [7]. Generally, with increased CD4 T cell levels, spontaneous clearing of the parasite takes place [8] and chronic diarrhea and cryptosporidial infection often resolves. Currently, there are more than 27 recognized species of *Cryptosporidium* infecting a wide variety of animals including humans [9-11]. 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 [12,13]. 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 [14,15]. 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 [16].
In Malaysia, the first cryptosporidiosis case was reported in 1984 [17]. So far, studies in Malaysia have only focused on determining the prevalence rate of cryptosporidiosis in HIV patients [18-22]. Molecular tools are very useful in tracking infection and contamination sources. Lim et al. [20] 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* subgenotype Ia, Ib, Id, Ie and If in HIV patients [20]. Moreover, another study targeting gp60 gene identified *C. parvum* IIa, IId and *C. hominis* Ia and Ib subgenotypes in Malaysian HIV patients [23]. There is little information on the distribution of *Cryptosporidium* species in Malaysia, and the susceptibilities of HIV-infected 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-infected individuals in order to determine their public health significance.

**Methods**

**Sample collection and microscopic examination**

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. Single stool samples from 346 patients were collected in sterile screw-capped fecal
containers with 2.5% potassium dichromate solution as a preservative and stored at 4°C prior to analysis.

Stool samples were subjected to coproscopic examination. Small portions of fecal samples were mixed with a drop of iodine on microscope slides and covered with a cover slip. Slides were then examined under 100X and 400 X magnifications to detect cysts and ova 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.

The group of HIV patients have also being diagnosed for the presence of other infections. **Extraction of genomic DNA from *Cryptosporidium* oocysts**

Microscopically positive *Cryptosporidium* samples were used for molecular characterization. Concentrated oocysts from fecal samples was acquired by mixing a small portion of the feces with 10 ml of distilled water and sieved 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) according to the manufacturer’s protocol. Briefly, IMS-isolated oocysts were resuspended in 180 µl of Buffer ATL provided with the kit, and underwent five consecutive cycles of freezing in liquid nitrogen for 1 min and thawing at 56 °C for 2 min,
with vortexing for 30 sec for every cycle. DNA was extracted with proteinase K and then purified using the DNA Mini kit. DNA template was stored at -20 ºC until further analysis.

**Amplification of Cryptosporidium DNA by nested-PCR**

A partial polymorphic region of SSU rRNA was amplified, according to Nichols *et al.*[14] in two stages, namely the primary reaction that amplified 655 to 667 bp fragments using a 27-mer forward primer (N-DIAGF2: 5’-CAA TTG GAG GGC AAG TCT GGT GCC AGC-3’) and a 26-mer reverse primer (N-DIAGR2: 5’-CCT TCC TAT GTC TGG ACC TGG TGA GT-3’) and the secondary reaction that amplified a 435 bp fragment using the forward primer (CPB-DIAGF: 5’-AAG CTC GTA GTT GGA TTT CTG-3’) and reverse primer (CPB-DIAGR: 5’- TAA GGT GCT GAA GGA GTA AGG-3’) that were previously developed by Johnson *et al.*[24].

The primary reaction was run in a total volume of 50 µl, containing 200 µM of each of the four deoxynucleoside triphosphates (dNTP) (Fermantas, cat. No. #R0192, Ontario, Canada), 0.2 µM of each primer N-DIAGF2/ N-DIAGR2 and CPB-DIAGF/R (Research Biolab, Singapore), 400 µg/ml BSA (New England Biolabs, cat. No. # B14, Ipswich, USA), 3.5 mM MgCl₂ (Fermentas, cat.no. # R0971, Ontario, Canada), 2.5 U *Taq* polymerase (New England Biolabs, cat. No. M0267L, Ipswich, USA), and 1X ThermoPol PCR buffer (New England Biolabs, cat. No. M0267L, Ipswich, USA). Two µl of DNA template was used in the primary PCR whereas 5 µl of the first PCR product was used as template in the secondary PCR. The secondary PCR reagent concentrations were the same as those used in the primary reaction as mentioned above. The cycling conditions were as follows; hot start at 95ºC for 5 min, followed by 35 cycles of denaturing for 30 sec at 94ºC, annealing for 1 min at 68ºC and extension for 30 sec at 72ºC, followed by a final extension at 72ºC for 10 min. The secondary PCR had a similar cycling condition except that the annealing temperature was only 60ºC. Positive (extracted DNA
of *C. parvum* oocysts purchased from Waterborne, Inc. New Orleans, USA) and negative (DNase-free water instead of DNA template) controls, were included in each amplification run. The PCR product was analyzed by electrophoresis in a 2% agarose gel and visualization of SYBR® Safe (Molecular Probes, Inc. USA) stained DNA was performed by ultraviolet light illumination of gels using a UV transilluminator.

**Sequencing and *Cryptosporidium* genotyping**

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 using the forward primer (CPB-DIAGF) and reverse primer (CPB-DIAGR) [24]. 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.

**Results**

Microscopic examination of all 346 fecal samples obtained from HIV patients indicated that the prevalence of *Cryptosporidium* infection 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%) 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) (Table 1). Four (9.3%)
patients were symptomatic 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 transmission, CD4 T cell counts, use of highly active antiretroviral 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”) (Table 1). No homosexual patients were found to have *Cryptosporidium* infection in the current study.

With regards to CD4 T cell count, there were 27 (79.4% of 34) patients who had CD4 counts < 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 (Table 1). CD4 T cell counts were further divided into various categories such as: 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$^3$ (Table 1).

In addition, 22 (64.7%) of the 34 *Cryptosporidium*-positive HIV patients also had co-infection of opportunistic infections (OIs). The most commonly identified opportunistic infections recorded in these 22 HIV patients were *Mycobacterium tuberculosis* infection (11, 50.0%), followed by cerebral toxoplasmosis (5, 22.7%), candidiasis and cryptococcosis (3 each, 13.6%), *Salmonella* septicaemia and *Pneumocystis 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 2). The most common type of
protozoa detected in Cryptosporidium-positive HIV patients was Entamoeba histolytica/dispar (41.9% of 43) followed by Isospora 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 2). The combination of mixed infections of Cryptosporidium with E. histolytica/dispar and A. lumbricoides was the most widespread among the HIV patients.

All microscopically Cryptosporidium-positive samples (i.e., 43 samples) were genetically analyzed using the nested-PCR protocol modified by Nichols et al.[14] from a previous protocol developed by Johnson et al.[24]. This protocol 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 Cryptosporidium specimens.

In order to determine the species of Cryptosporidium isolates from the HIV patients, all 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 3). 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.
Detailed analysis highlighted that the majority of the 27 HIV patients infected with *C. parvum* were adults (92.5%, 25), and that 24 (88.8%) were males and 3 (11.1%) were females. Most of them were Malay (37.0%, 10), IVDU (63.0%, 17), CD4 count < 50 cells/mm\(^3\) (55.5%, 15), undergoing HAART (55.5%), 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). 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), *I. 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 3).

The two patients infected with *C. 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 3). This patient was also co-infected with OIs, mainly *Herpes simplex* and Kaposi’s sarcoma (Table 3).

The two patients infected with *C. meleagridis* were Malay adult male. 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 3). 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 *C. felis* was Malay male; who did not disclosed his mode of HIV transmission. This patient had CD4 count of < 50 cells/mm\(^3\) and was not on HAART therapy. He also had cerebral toxoplasmosis and *A. lumbricoides* infection (Table 3).

**Discussion**
In this study, we found a high prevalence 12.4% (43) of Cryptosporidium oocysts in fecal samples from HIV-patients in Malaysia. These results are in accordance with previous studies carried out among HIV-positive IVDU patients in Malaysia [18], and in hospitalized HIV patients in Kota Bharu [22]. However, another Malaysian study reported a much lower prevalence (3%) of cryptosporidiosis among HIV patients [19]. In Malaysia, there have been studies conducted to determine the prevalence of cryptosporidiosis in children and in immune-compromised patients, such as those with HIV and cancer. In a study of hospitalized patients, the infection rate of cryptosporidiosis was 0.3% [25]. Epidemiological surveys carried out in various parts of the world revealed that the prevalence rate of cryptosporidiosis ranged from 3-50% [26-28]. The prevalence of cryptosporidiosis in HIV patients also varies among these studies, depending on where they were conducted, age of the patients, the stage of disease (HIV/AIDS) and the laboratory methods used [29]. Results of the present study are in concordance with the studies carried out in other parts of the world such as Iran [30,31], Brazil [32], India [8,33,34], Thailand [1,35], Cambodia [29], Ethiopia [28] and Nigeria [36].

Many studies have highlighted Cryptosporidium as the predominant pathogen with significant association to diarrheal illness as well as its association with asymptomatic cases among HIV positive individuals. The occurrence of Cryptosporidium in both symptomatic and asymptomatic cases indicated a high risk of infection of this parasite [37]. The present study revealed predominantly asymptomatic HIV carriers of Cryptosporidium. Latent infections can be reactivated and may lead to recurrence later when the immunity of patient diminishes further. Adesiji et al. [36] reported a high prevalence rate (52.7%) of Cryptosporidium infection in Nigerian HIV patients with diarrhea. The reason for the large difference in prevalence may be related to the fact that HIV opportunistic infections, cryptosporidiosis inclusive, tend to vary
from one locality to another and from one country to the other depending on the level of
contamination of water, foodstuff and contacts with animals, which are important factors in
dissemination of this parasite.

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 other protozoan
parasites detected in Cryptosporidium-positive patients consisted of E. histolytica/dispar, I. belli,
G. duodenalis and C. cayetanensis. Helminths observed included A. lumbricoides, T. trichiura
and hookworm in HIV patients. The most common multi-parasitic co-infections observed among
these patients were with E. histolytica/dispar and A. lumbricoides. Aseefa et al. [28] also
highlighted that 49.5% of HIV patients harbour single and multiple infections (polyparasitism) of
Cryptosporidium and I. belli. Our results are in agreement with the findings of Adesiji et al. [36]
which detected a high prevalence of T. trichiura, A. lumbricoides, and G. duodenalis in Nigerian
Cryptosporidium-positive HIV patients. E. histolytica and G. duodenalis, along with
Cryptosporidium species and I. belli have been reported as the most common causes of diarrhea
in HIV patients from India [38].

Our results indicate that adult patients harbour greater diversity of other intestinal
parasites as compared to children (those 12 years and below). However, there is limited data
available on this group. In one study in Malaysia, Ludin et al. [39] studied 863 stool samples
with diarrhea and acute gastroenteritis in the pediatric ward, Penang General Hospital and found
only 4.3% were positive for Cryptosporidium and the prevalence was 2.39% higher in children
with diarrhea and vomiting than in children with acute gastroenteritis alone (0.8%). Another
study conducted in hospitalized children in Kota Bharu by Menon et al.[40] showed that only 1
of the 109 (0.9%) children with acute diarrhea was positive for *C. parvum* by microscopy and PCR, whereas a 2-month old girl known to be HIV positive, had chronic diarrhea lasting for 2 months but her stools were negative for *C. parvum*. 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 [21,42]. The variation of these finding with reports from the industrialized countries could be due to differences in the study population and also 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 of parasitic infections between males and females. In male *Cryptosporidium*-positive HIV patients, protozoa infections were more common compared to helminths. However in female *Cryptosporidium* HIV patients, only single protozoa infections (25%, 1 of 4) were founds, neither helminths nor any mixed infections of protozoa and helminths were detected. Zali *et al.* [43] noted no significant difference between males and females (18.5% versus 18.1%, respectively; p > 0.05). In addition, Srisuphanunt *et al.* [35] also reported no statistically significant difference between the socio-demographic characteristics of the patients with and without *Cryptosporidium*. This may be due to the fact that most of the HIV patients in Asia-Pacific countries are of low socioeconomic status [35]. The present findings suggested that the prevalence of cryptosporidiosis may vary by ethnic groups, depending on the degree of their immune status. Dwivedi *et al.* [34] reported that age and gender were found to be non-significant factors responsible for parasitic infestations.

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
hookworm (for IVDU) and *Cyclospora* (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.

Based on CD4 count, it was noted that *Cryptosporidium*-positive patients with CD4
counts < 200 cells/mm$^3$ had higher prevalences of all types of other parasites. There is a wealth
of information documenting the correlation between CD4 count < 200 cells/mm$^3$ and

symptomatic/asymptomatic cryptosporidiosis [26,34,38,44]. Detailed analysis based on different
categories of CD4 T cell count highlighted that *Cryptosporidium*-positive patients with CD4
counts of $\leq$ 50 cells/mm$^3$ were infected with many types of other parasites such as *E.

*histolytica/dispar, I. belli, G. duodenalis, Cyclospora catayanensis, A. lumbricoides, T. trichiuria*

and hookworm. This is seen less in *Cryptosporidium*-positive patients with higher CD4 counts.

Those with 201-400 cells/mm$^3$ only harboured *E. histolytica/dispar, I. belli, G. duodenalis* and *A.

*lumbricoides* whilst those *Cryptosporidium*-positive patients with > 400 cells/mm$^3$ only had *E.

*histolytica/dispar* and *A. lumbricoides* co-infections.

Researchers from Iran determined the same pattern of parasitic infection in different
categories of CD4 cell counts on the basis of diarrheal status (i.e., acute and chronic) 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).

Similarly, HIV individuals in Indonesia with CD4 cell counts of $\leq$ 50/mm$^3$ were observed to
harbour more types of intestinal parasites such as *Blastocystis, Cyclospora, Cryptosporidium,

*Giardia, Entamoeba* and *Ascaris* compared to those with CD4 counts of 51-100 cells/mm$^3$ and

101-200 cells/mm$^3$ [45]. As with previous studies [8,28,46] parasites associated with HIV
patients were more likely encountered as the CD4 count dropped below 200 cells/mm$^3$. This may
be due to immunocompromised patients were more susceptible to parasitic infections or were
unable to clear infection once established.

The clinical profile of *Cryptosporidium*-positive HIV patients included a wide range of
other opportunistic infections (OIs) including *Mycobacterium tuberculosis*, cerebral
toxoplasmosis, candidiasis, cryptococcosis, *Salmonella* septicaemia and *Pneumocystis carinii*
pneumonia. In India, tuberculosis is the most common opportunistic infection in HIV patients
[47]. In Portugal, HIV patients with *Cryptosporidium* infection were also observed to have
tuberculosis (19%), *Pneumocystis carinii* pneumonia (14%), *Salmonella* sepsis (6%), isosporiasis
(3%), toxoplasmic encephalitis (3%), leishmaniasis (3%) and Kaposi’s sarcoma (3%) [48],
whereas in Thailand, 59% of 22 *Cryptosporidium*-positive HIV patients also had tuberculosis
(38.5%), *Pneumocystis carinii* pneumonia (23.1%), cryptococcal meningitis and cryptococcemia
(15.4%), and penicilliosis (7.7%) [49]. Adesiji *et al.* [36] reported an association of
*Cryptosporidium* with *cytomegalovirus* infection, and suggested that this co-infection
underscores the importance of the identification of concomitant gastrointestinal infection in HIV
patients. Based on these findings, the only parameter unequivocally related to opportunistic
infections in HIV patients is the immune status. HAART regimen may help to reduce
acquisitions of these opportunistic infections in immune-compromised patients.

Recently, advances in molecular techniques have increased the sensitivity of
*Cryptosporidium* detection, as well as allowing for a simultaneous genetic characterization of
isolates obtained from HIV/AIDS patients. The most common PCR target gene utilised in
molecular studies is SSU rRNA. Lim *et al.* [20] 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), followed by *C. hominis* (six isolates), *C. meleagridis* (two isolates) and *C. felis* (one isolate). Another study in Malaysia reported only *C. parvum* in 9 HIV patients [22] and none of the other species were encountered in that study. We documented the distribution of cryptosporidial species and subgenotypes in HIV-infected individuals in Malaysia. 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 genotype analysis showed HIV patients were infected with *C. parvum* IIa and IIId genotypes and *C. hominis* Ia and Ib genotypes denoting the possibility of zoonotic as well as anthroponotic transmissions of cryptosporidiosis in these individuals [23].

The detection of a wide range of *Cryptosporidium* species in a population is not uncommon, although *C. parvum* is usually the predominant and more frequent species infecting HIV patients [31,48,50-52]. HIV patients are known to be susceptible to a broad spectrum of species and genotypes of *Cryptosporidium* [53]. In Thailand, anthroponotic and zoonotic species (i.e., *C. parvum, C. hominis, C. meleagridis, C. felis*, and *C. canis*) were found to be almost equally common in HIV-infected individuals [54]. Xiao et al. [15] reported five different types of *Cryptosporidium* species from among Peruvian HIV-infected patients, including *C. parvum, C. hominis, C. meleagridis, C. felis, and C. muris*, whereas Cama et al. [55] identified six species (*C. parvum, C. hominis, C. meleagridis, C. felis, C. muris* and *C. suis*) in Peruvian HIV patients, but the majority of the patients were infected with *C. hominis*. Another study from Brazil describes several *Cryptosporidium* species found in HIV infected patients [56]. Interestingly, *C. felis* was found to be almost as common as *C. parvum*. These findings indicated that, in Brazil, the cat adapted *C. felis* may play a potential role in the zoonotic transmission of cryptosporidiosis.
whereas the anthroponotic transmission of cryptosporidiosis caused by *C. hominis* seems to
predominate [56].

Among the zoonotic species detected in this 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 *Cryptosporidium* species (*C. parvum* versus *C. hominis*) was observed in children
[31]. The current results are in agreement with this study as they showed that *Cryptosporidium*
species distribution in adult patients and children (those of 12 years and below) were similar.
However, these findings differ from studies conducted in Peru, Thailand, Malawi, Uganda,
Kenya, South Africa, Tunisia and South India, which showed a predominance of *C. hominis* in
children or HIV positive adults [53,57-64]. It is obvious based on these studies that the
distribution of the infecting species of *Cryptosporidium* varies according to the type of
population, age of subjects, and level of immune status, season, and the geographic location.

Transmission routes for the rare *Cryptosporidium* species are unclear. This is because
human infection with those *Cryptosporidium* species is less common, and the principal
transmission route for these parasites is likely through direct contact with infected animals [65].
For instance, *C. meleagridis* normally infects turkeys [66]. However, this species has been
confirmed in an Indian ring-necked parrot, a common aviary bird the world over [67]. As HIV
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 [67].
Studies have shown that HIV patients have acquired *C. felis* infections from their pets, especially cats [51,65,67]. However, in the present study, no data on animal contact was available from HIV patients; therefore concrete inference could not be made. Nonetheless in Malaysia, it is common for households to have cats as pets and this could be a source of infection in these patients. Thus, the significance of this mechanism of transmission warrants further investigations.

**Conclusion**

Given that the presence of infection due to zoonotic *Cryptosporidium* species in HIV patients is significant, it is crucial that immunocompromised patients are being informed on the potential of acquiring cryptosporidiosis from infected animals and contaminated water. 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.

**Competing interests**

The authors have declared that no competing interests exist.

**Author Contributions**

AI, JS and YALL planned and designed the protocols. JS and YALL supervised all the laboratory work. AI was involved in all phases of the study, including data collection, performed the experiments and interpretation. AI, BLH and YALL were involved in collection and laboratory examination of samples, AI prepared the first draft of the manuscript and YALL, BD
and JS revised the manuscript critically. All authors read and approved the final version of the manuscript.

Acknowledgments

The authors would like to thank the staff of Hospital Sungai Buloh, Selangor; University of Malaya Medical Centre, Kuala Lumpur and Hospital Raja Zainab Perempuan II, Kelantan, for their kind help and cooperation. This study was funded by the research grant from University of Malaya-50603 Kuala Lumpur, Malaysia (Research grant No. PS007/2008B and PS203-2010A).

Authors details

1Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia. 2Microbiology 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. 3Infectious Disease Unit, Department of Medicine, Hospital Sungai Buloh, 47000 Sungai Buloh, Selangor Darul Ehsan, Malaysia

References


Table 1 Association of demographic and clinical features of 43 HIV patients infected with Cryptosporidium spp.

<table>
<thead>
<tr>
<th></th>
<th>Infected (n)</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (Years)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Children (≤ 12)</td>
<td>4</td>
<td>9.3</td>
</tr>
<tr>
<td>Adults (22 to 54)</td>
<td>39</td>
<td>90.7</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>39</td>
<td>90.7</td>
</tr>
<tr>
<td>Female</td>
<td>4</td>
<td>9.3</td>
</tr>
<tr>
<td><strong>Ethnic Groups</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malay</td>
<td>24</td>
<td>55.8</td>
</tr>
<tr>
<td>Chinese</td>
<td>10</td>
<td>23.3</td>
</tr>
<tr>
<td>Indians</td>
<td>4</td>
<td>9.3</td>
</tr>
<tr>
<td>Foreigners</td>
<td>5</td>
<td>11.6</td>
</tr>
<tr>
<td><strong>Diarrhea</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>4</td>
<td>9.3</td>
</tr>
<tr>
<td>No</td>
<td>39</td>
<td>90.7</td>
</tr>
<tr>
<td><strong>Based on 34 patients</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mode of Transmission</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVDU</td>
<td>20</td>
<td>58.8</td>
</tr>
<tr>
<td>Heterosexual</td>
<td>7</td>
<td>20.6</td>
</tr>
<tr>
<td>Unknown</td>
<td>7</td>
<td>20.6</td>
</tr>
<tr>
<td><strong>CD4 counts/ mm$^3$</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 200</td>
<td>27</td>
<td>79.4</td>
</tr>
<tr>
<td>&gt; 200</td>
<td>6</td>
<td>17.6</td>
</tr>
<tr>
<td>NA</td>
<td>1</td>
<td>2.9</td>
</tr>
<tr>
<td><strong>Categories of CD4 counts/ mm$^3$</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-50</td>
<td>18</td>
<td>52.9</td>
</tr>
<tr>
<td>51-100</td>
<td>3</td>
<td>8.8</td>
</tr>
<tr>
<td>101-200</td>
<td>6</td>
<td>17.6</td>
</tr>
<tr>
<td>201-400</td>
<td>4</td>
<td>11.8</td>
</tr>
<tr>
<td>&gt; 400</td>
<td>2</td>
<td>5.9</td>
</tr>
<tr>
<td>NA</td>
<td>1</td>
<td>2.9</td>
</tr>
<tr>
<td><strong>Opportunistic infections</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>22</td>
<td>64.7</td>
</tr>
<tr>
<td>No</td>
<td>12</td>
<td>35.3</td>
</tr>
</tbody>
</table>

*Clinical data available only for 34 patients (10 patients did not provide consent for their clinical records to be accessed).

NA= Not available (CD4 count was not conducted for this patient).
Table 2 Other intestinal parasites detected in *Cryptosporidium*-positive HIV patients (n = 43)

<table>
<thead>
<tr>
<th>Parasites</th>
<th>No. infected</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protozoa</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Entamoeba histolytica/dispar</em></td>
<td>18</td>
<td>41.9</td>
</tr>
<tr>
<td><em>Isospora belli</em></td>
<td>8</td>
<td>18.6</td>
</tr>
<tr>
<td><em>Giardia duodenalis</em></td>
<td>6</td>
<td>14.1</td>
</tr>
<tr>
<td><em>Cyclospora cayetanensis</em></td>
<td>2</td>
<td>4.7</td>
</tr>
<tr>
<td><strong>Helminths</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ascaris lumbricoides</em></td>
<td>11</td>
<td>25.6</td>
</tr>
<tr>
<td><em>Trichurus trichiura</em></td>
<td>4</td>
<td>9.3</td>
</tr>
<tr>
<td>Hookworm</td>
<td>1</td>
<td>2.3</td>
</tr>
<tr>
<td><strong>Intestinal parasitic infections IPIs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cryptosporidium</em> infection (Single infection)*</td>
<td>18</td>
<td>41.9</td>
</tr>
<tr>
<td><em>Cryptosporidium</em> + Protozoa infections</td>
<td>10</td>
<td>23.3</td>
</tr>
<tr>
<td><em>Cryptosporidium</em> + Protozoa + Helminth infections</td>
<td>15</td>
<td>34.9</td>
</tr>
</tbody>
</table>

Table 3 Demographic, clinical characterization and species distribution of *Cryptosporidium* in HIV patients (n = 32)

<table>
<thead>
<tr>
<th></th>
<th>C. <em>parvum</em> n = 27 (%)</th>
<th>C. <em>hominis</em> n = 2 (%)</th>
<th>C. <em>meleagridis</em> n = 2 (%)</th>
<th>C. <em>felis</em> n = 1 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Children (&lt; 12)</td>
<td>2(7.4)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Adults (22 to 54)</td>
<td>25(92.5)</td>
<td>2 (100.0)</td>
<td>2 (100.0)</td>
<td>1 (100.0)</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>24(88.8)</td>
<td>2 (100.0)</td>
<td>2 (100.0)</td>
<td>1 (100.0)</td>
</tr>
<tr>
<td>Female</td>
<td>3(11.1)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Ethnic Groups</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malay</td>
<td>10 (37.0)</td>
<td>1 (50.0)</td>
<td>2 (100.0)</td>
<td>1 (100.0)</td>
</tr>
<tr>
<td>Chinese</td>
<td>9 (33.3)</td>
<td>1 (50.0)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Indians</td>
<td>4 (14.8)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Foreigners</td>
<td>4 (14.8)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Mode of Transmission</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterosexual</td>
<td>3 (11.1)</td>
<td>1 (50.0)</td>
<td>1 (50.0)</td>
<td>-</td>
</tr>
<tr>
<td>IVDU</td>
<td>17 (63.0)</td>
<td>1 (50.0)</td>
<td>1 (50.0)</td>
<td>-</td>
</tr>
<tr>
<td>Unknown</td>
<td>6 (22.2)</td>
<td>-</td>
<td>-</td>
<td>1 (100.0)</td>
</tr>
<tr>
<td>NA</td>
<td>1 (3.7)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>CD4 counts/ mm$^3$</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-50</td>
<td>15 (55.5)</td>
<td>2 (100.0)</td>
<td>-</td>
<td>1 (100.0)</td>
</tr>
<tr>
<td>51-100</td>
<td>2 (7.4)</td>
<td>-</td>
<td>1 (50.0)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>101-200</td>
<td>4 (14.8)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>201-400</td>
<td>4 (14.8)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>&gt; 400</td>
<td>1 (3.7)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NA</td>
<td>1 (3.7)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Intestinal parasites**

**Protozoa**

*Entamoeba histolytica/dispar*<br>15 (55.5) - 1 (50.0) -

*Isospora belli*<br>8 (29.6) - - -

*Giardia duodenalis*<br>5 (18.5) 1 (50.0) - -

*Cyclospora cayetanensis*<br>1 (3.7) - - -

**Helminths**

*Ascaris lumbricoides*<br>8 (29.6) 1 (50.0) - 1 (50.0)

*Trichuris trichiuria*<br>3 (11.1) - - -

*Hookworm*<br>1 (3.7) - - -

**Opportunistic infections**

*Yes*<br>18 (66.6) 1 (50.0) 1 (50.0) 0

*No*<br>8 (29.6) 1 (50.0) 1 (50.0) 1 (100.0)

*NA*<br>1 (3.7) - - -

*Mycobacterium tuberculosis*<br>11 (40.7) - - -

*Mycobacterium*: disseminated or extrapulmonary<br>3 (11.1) - - -

*Salmonella septicemia*<br>2 (7.4) - - -

*Candidiasis*<br>2 (7.4) - - -

*Pneumocystis carinii pneumonia*<br>2 (7.4) - - -

*Cryptococcosis*<br>2 (7.4) - 1 (50.0) -

*Histoplasmosis*<br>1 (3.7) - - -

*Herpes simplex*<br>- 1 (50) - -

*Cerebral Toxoplasmosis*<br>4 (14.8) - - 1 (100)

*Primary lymphoma of brain*<br>1 (3.7) - - -

*Kaposi’s sarcoma*<br>- 1 (50) - -

*Wasting syndrome*<br>1 (3.7) - - -

*HIV-related encephalopathy*<br>1 (3.7) - - -

NA: Not Available