**Cryptosporidium species** from human immunodeficiency—infected patients with chronic diarrhea in Jakarta, Indonesia

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**Abstract**

**Purpose:** Cryptosporidium is an opportunistic parasite that manifests as chronic and severe diarrhea in the immune-compromised subject. We investigated the species of Cryptosporidium to understand the epidemiology, mode of transmission, response to treatment, and prevention.

**Methods:** Polymerase chain reaction/restriction fragment length polymorphism of the 18 S rRNA gene and sequencing were performed on 41 Cryptosporidium-positive stools from 36 patients with HIV/AIDS, which comprised 36 pretreatment stools and 5 stools after treatment with Paromomycin.

**Results:** C. hominis, C. meleagridis, C. felis, and C. parvum were detected; 28 of 36 (77.8%) patients were infected with C. hominis and two (5.5%) patients with multiple species of Cryptosporidium. Treatment with Paromomycin resulted in different outcomes, perhaps because patients harbored other intestinal parasitic infections.

**Conclusions:** Multiple infection with various Cryptosporidium species in the presence of other intestinal parasites occurs in patients with HIV/AIDS suffering from chronic diarrhea who are severely immune-compromised. Common transmission of Cryptosporidium is anthropoontic.

**Introduction**

The intracellular, protozoan parasite, Cryptosporidium, is found worldwide. The disease, cryptosporidiosis, is one of the most important and common causes of acute enteric protozoal diarrhea, which leads to economically significant morbidity and mortality in humans worldwide. In immuno-competent individuals, cryptosporidiosis is self-limiting, presenting clinically with mild gastrointestinal symptoms [1], but in immune-compromised individuals, cryptosporidiosis can be a serious, life-threatening condition. Immune-compromised individuals, particularly those with human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS), and who are not on highly active antiretroviral therapy (ART), can suffer intractable, voluminous secretory diarrhea accompanied by severe abdominal cramps, weight loss, anorexia, malaise, low-grade fever, and wasting, which can be fatal [2], largely because of the lack of effective chemotherapy [3].

Cryptosporidiosis can occur at any time during the course of HIV infection. However, severe and persistent disease correlates well with CD4 counts of less than 180 cells/mm\(^3\). In one study, only 5 of 39 (13%) patients infected with Cryptosporidium parvum and with CD4 counts of less than 180 cells/mm\(^3\) had self-limiting disease, whereas all 8 patients with CD4 counts of greater than 180 cells/mm\(^3\) had infections that cleared and did not relapse during a follow-up period of 1–24 months [4].

At least seven Cryptosporidium species have been detected in immune-compromised individuals [5], but as only a few studies have investigated human cryptosporidiosis in Indonesia [6–9], there is a lack of understanding on the importance of Cryptosporidium species and genotypes infecting these individuals and the epidemiology of this disease. Our previous study identified Cryptosporidium oocysts in 11.9% (n = 318) of HIV cases with CD4 counts less than 200 cells/mm\(^3\), determined by modified acid fast staining (AFS) from unconcentrated stools [6]. Here, we report on the specieses of Cryptosporidium infected the HIV/AIDS cases with chronic diarrhea in Jakarta with further parasitological evaluation on cases receiving treatment with Paromomycin.

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http://dx.doi.org/10.1016/j.annepidem.2013.07.019
Materials and methods

Study population

This study is part of a wider investigation on the profile of parasitic infections amongst immune-compromised subjects. A cross sectional study design was used. The study group comprised HIV AIDS patients diagnosed with chronic diarrhea. Stools were submitted to the Laboratory of the Department of Parasitology, Faculty of Medicine, Universitas Indonesia during a 29-months period (November 2004–March 2007). The profile of parasitic infections among those HIV-infected patients had been previously reported [6]. Samples were not randomised. To summarize, a total of 318 patients ages 5 months to 55 years, with laboratory-confirmed HIV and diarrhea for longer than 4 weeks were identified for inclusion in the study. Whenever the presence of Cryptosporidium oocysts was detected by modified AFS, the physician in charge was informed with a request for a second stool specimen to be forwarded for evaluation after treatment. Paromomycin (3 × 500 mg for 28 days) was given to patients with Cryptosporidium infections only, without co-infection with other intestinal parasites. All patients diagnosed with HIV and with a CD4 count of less than 200 cells/mm³ were administered co-trimoxazole as a prophylaxis against Pneumocystis and other susceptible opportunistic infections such as Toxoplasma, Cyclospora, and Isospora. ART was administered after confirmation of an HIV diagnosis, which was usually at a later stage because the patients initially came to the hospital as the result of the severe diarrhea, which was chronic and persistent. Ethical clearance was provided by the Ethics and Research Committee, Faculty of Medicine, Universitas Indonesia.

Parasitology

All samples were examined directly by wet mount with a drop of Lugol's iodine solution to identify any protozoan cyst, trophozoites, and the worm eggs. For Blastocystis hominis, detection was done by direct stool examination and confirmed by culture using Jones' medium for 24 hours and incubated at 37°C [6]. Identification of Cryptosporidium oocysts in fecal samples was performed by the AFS method from uncentranted stools. On the basis of the number of oocyst detected, the AFS results were classified as negative, +1, +2, or +3 [10]. All samples positive for Cryptosporidium or other parasites were stored in 2.5% potassium dichromate solution at 4°C for up to 2 years before we performed DNA extraction.

DNA extraction

Cryptosporidium oocysts were retrieved from oocysts-positive stool samples with a water-ether procedure adapted from Bukhari and Smith, [11] by Nichols et al [12] for extracting DNA from stool samples in 1.5-ml microcentrifuge tubes. The described method [12] was followed with the exception of the introduction of one initial wash of the fecal material (200 μL of wet or semisolid stools) in 1.2 mL of sterile double-distilled water by centrifugation (14,000g for 1 minute at room temperature) to remove the potassium dichromate. DNA extraction was performed by disrupting the oocysts suspended in approximately 100 μL of lysis buffer (50 mM Tris-HCl, pH 8.5; 1 mM ethylenediamine tetraacetic acid; 0.5% sodium dodecyl sulfate) by 15 consecutive cycles of freezing in liquid nitrogen (1 min) and thawing at 65°C in a water bath (1 min) [12]. Samples were digested with proteinase K (200 μg/mL) at 55°C for 3 h, incubated at 90°C for 20 min to inactivate the proteinase K and cooled on ice for 1 min prior to centrifugation at 14,000g for 5 minutes at room temperature [12]. The supernatants were transferred to clean sterile tubes and kept at −20°C until used.

PCR-restriction fragment length polymorphism (RFLP)

Cryptosporidium species was determined by direct PCR-RFLP by targeting the 18 S rRNA gene [13]. All PCR amplifications were performed in the MJ Research thermocycler (St. Bruno, Canada) with 200-μL thin-walled tubes and the following polymerase chain reaction (PCR) reagents: 200 nM of the forward and reverse primers, DNase free water, 1X PCR buffer, 200 μM of each of the four dNTPs, BSA at 4 mg/mL, 2% Tween 20, 2.5 mM MgCl₂ and Taq Polymerase (0.5 U) final concentration in 50-μL reaction. Confirmation of PCR results was carried out at the Scottish Parasite Diagnostic Laboratory with the use of two 18 S nested-PCR assays as described previously [14–16]. RFLP analyses were performed by digestion of the amplicons with restriction enzymes followed by visual observation of the fragments by gel electrophoresis and ethidium bromide staining [15]. Secondary amplicons (approximately 435 bp) obtained from the nested PCR technique of Nichols et al (2003) was digested simultaneously with Asel and Dral (Invitrogen/New England Biolabs, Ipswich, MA), whereas the nested PCR yielding secondary amplicons of approximately 847 bp in length [16] was digested separately with Asel and SspI (Invitrogen) and the results interpreted according to Xiao et al [16]. Sequencing was performed when RFLP analyses failed to diagnose the species.

Results

There were 41 stool samples from 36 patients (including five posttreatment stools) positive for Cryptosporidium sp. oocysts by AFS that were further analyzed by the use of PCR-RFLP for species determination. The patients’ ages ranged from 3.5 to 55 years; two children were 3.5 and 5 years of age, and the rest of the patients were older than 20 years of age. All patients were male, except one adult female, and had CD4+ cells counts of less than 50 cells/mm³. Twenty-eight of 36 patients (77.7%) harbored a single species (Cryptosporidium hominis, n = 23; Cryptosporidium felis, n = 3; Cryptosporidium meleagridis, n = 2; Table 1). Two patients were found to be infected with more than one species of Cryptosporidium. One patient had a mixed infection of C. hominis and C. meleagridis and the other a mixture of three species (C. hominis, C. meleagridis, and C. parvum). Six patients showed negative result by PCR-RFLP.

There were five patients from whom posttreatment stool samples were collected for parasitological examination because of persistent diarrhea. The interval between the pre- and posttreatment samples varied between 1 and 5 months. On the follow-up samples, the stools were again examined by direct smear, culture for B. hominis, and AFS from uncentrated stools to detect any helminths ova/larvae and protozoan cysts/oocysts/trophozoites. The results of AFS staining on follow-up stools varied from low (+1) to moderate (+2) numbers of oocysts per field of view (Table 2).

Table 1

<table>
<thead>
<tr>
<th>Type of infection</th>
<th>No. patients (%)</th>
<th>Cryptosporidium species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single</td>
<td>28 (77.7)</td>
<td>23 with C. hominis,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 with C. felis,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 with C. meleagridis,</td>
</tr>
<tr>
<td>Double</td>
<td>1 (2.7)</td>
<td>C. hominis, C. meleagridis</td>
</tr>
<tr>
<td>Triple</td>
<td>1 (2.7)</td>
<td>C. hominis, C. meleagridis, C. parvum</td>
</tr>
<tr>
<td>Negative</td>
<td>6 (16.7)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>36 (100)</td>
<td></td>
</tr>
</tbody>
</table>

AIDS = acquired immunodeficiency syndrome; HIV = human immunodeficiency virus; PCR = polymerase chain reaction; RFLP = restriction fragment length polymorphism.
Table 2
Cryptosporidium infection in follow-up patients detected by PCR-RFLP and AFS

<table>
<thead>
<tr>
<th>Code</th>
<th>First visit</th>
<th>Second visit</th>
<th>AFS</th>
<th>Duration between visits</th>
<th>Parasite infections (Microscopic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>001</td>
<td>C. hominis</td>
<td>Negative</td>
<td>+1/1</td>
<td>5 months</td>
<td>Cryptosporidium sp</td>
</tr>
<tr>
<td>002</td>
<td>C. hominis</td>
<td>Negative</td>
<td>+1/1</td>
<td>2 months</td>
<td>Cryptosporidium sp</td>
</tr>
<tr>
<td>003</td>
<td>C. hominis</td>
<td>C. hominis</td>
<td>+2/1</td>
<td>1 month</td>
<td>Cryptosporidium sp</td>
</tr>
<tr>
<td>004</td>
<td>C. meleagridis</td>
<td>C. meleagridis</td>
<td>−/−2</td>
<td>3 months</td>
<td>Giardia intestinalis</td>
</tr>
<tr>
<td>005</td>
<td>Negative</td>
<td>Negative</td>
<td>+1/1</td>
<td>2 months</td>
<td>Cryptosporidium sp</td>
</tr>
</tbody>
</table>

AFS = acid fast staining; PCR = polymerase chain reaction; RFLP = restriction fragment length polymorphism.

We noted discrepancies between the results of PCR-RFLP and AFS staining: (1) There were six samples that were positive by AFS and, hence, were negative on PCR-RFLP. (2) Three follow-up samples that were negative for Cryptosporidium spp. by PCR-RFLP were found to be positive (+1) by microscopy/AFS (samples no. 001, 002, and 005). This may be due to the following reasons: (i) false-positive reporting of the presence of Cryptosporidium oocysts in fecal samples from either the presence of other AFS microorganisms of a similar size and shape as Cryptosporidium oocysts or the misidentification of debris in the stained stool for Cryptosporidium oocysts because the use of unconcentrated stool on AFS and PCR-RFLP does not detect any DNA in the microorganism, causing delay in the treatment. (ii) The presence of PCR-inhibitory substances in the DNA samples. (iii) Insufficient DNA for PCR amplification.

In contrast, patient no. 004 was found upon initial examination of the stool sample to be negative by AFS but positive by PCR-RFLP (C. meleagridis). The first stool from patient no.004 was diagnosed with severe giardiasis; the doctor was notified and subsequently sent another stool after metronidazol treatment at which time, Cryptosporidium was detected and there was less Giardia intestinalis cysts. Since any positive stool was always kept in the fridge, the PCR of case no. 004 was done also by including the pre-treatment stool. Dominant Giardia cyst in the first stool made the microscopist fail to identify any Cryptosporidium oocyst from unconcentrated stool specimen however the molecular technique was successful.

The stools from four follow-up patients also contained other gastroenteric protozoan parasites ( Blastocystis hominis and Giardia intestinalis). In the presence of concurrent pathogenic parasitic protozoa and mixed infection by different Cryptosporidium species it is impossible to assign the clinical manifestations expressed by these patients to one particular pathogen or species.

Table 2 also showed the treatment results of Cryptosporidium with Paromomycin, suggested that there was no effect of treatment, as was seen by microscopic examination (AFS); however, the PCR-RFLP did not detect any Cryptosporidium DNA in the first two patients (Table 2), suggesting possibility of empty oocysts seen on microscope or PCR inhibition.

Discussion

After the emergence of HIV epidemic, as a developing country, Indonesia has a lot to do to manage this problem in addition to other endemic infectious diseases. Cryptosporidium spp. is one of the opportunistic parasites commonly coinfected patients with HIV and manifests as chronic and sometimes profuse diarrhea. This study found four species of Cryptosporidium among the studied HIV population in Indonesia with C. hominis being the dominant one, suggesting that the transmission is mainly human to human with cats and chicken or birds as other sources of infection. This study confirms other reports on the species of Cryptosporidium commonly infecting humans, in particular the HIV group of C. hominis, C. parvum, C. meleagridis, and C. felis [5,17–21]. However, the use of unconcentrated stools prevents the finding of more Cryptosporidium coinfections in this population.

It is understandable in this study that human-to-human (anthropogenic) transmission is the main route of Cryptosporidium infection among the HIV population because all patients involved in this study originated from Jakarta, the capital, and its surrounding, urban areas. Cats and rats are common animals easily found in housing complex area in Jakarta, and quite often chickens farming or birds and dogs are pets. The finding could be different if samples were from rural area or from particular working groups such dairy farmers, which allow also zoonotic transmission and other non-human Cryptosporidium species to occur [22,23].

Because there are very few studies on Cryptosporidium among the Indonesian population [6–9] and none on the genotyping, this finding should raise awareness in the management and care of HIV patients with particular interest on the prevention of transmission of Cryptosporidium from patients to the care givers within the hospital, family, and in the community. The presence of cats and birds as pets/prey roaming around and chicken farming should be treated cautiously to prevent the zoonotic transmission and multi-species of Cryptosporidium and other intestinal parasitic infection, which is common among patients with HIV AIDS [6] and also was observed in two patients in this study.

After treatment with antiparasitic drugs, the patients had fewer episodes of diarrhea, but it did not completely resolve. The persistence of diarrhea despite the administration of Paromomycin could be interpreted either a result of the ineffectiveness of the drug in patients with a defective immune system (all patients had CD4 counts < 50 cells/mm³) or the result of coinfection with other intestinal parasites that were not effectively eliminated or under-diagnosed Cryptosporidium infection attributable to the lack of sensitivity of the AFS method, causing delay in the treatment. Patients in this study also received antiretroviral treatment at a late stage of disease because of the delay in visiting the hospital to seek help. It is common in Indonesia for HIV patients to hide their clinical problems, to be afraid to declare their HIV status, and to come to the hospital at a late stage when their immune status had been highly compromised and they had acquired several opportunistic infecions. The consequences included treatment failure with anti-parasitic agents. Efforts have been made recently to increase awareness among the patients with HIV on opportunistic infections and encourage them to visit the hospital or HIV clinics at very early stage for anti retroviral treatment.

The effect of Paromomycin to treat cryptosporidiosis in AIDS patients has been shown previously to have an impact in ameliorating symptoms by reducing the number of stools per day and
improving weight gain, but not of cure [24]. Therefore, the partial effect of Paromomycin observed in the small number of patients treated in this study was expected. ART is currently the most effective way of controlling the symptoms of cryptosporidiosis in AIDS patients [25]. However, its application in developing countries is compounded by difficulties, some of them expressed earlier in this discussion.

We can conclude from our study that multiple infection of Cryptosporidium species as well as with other intestinal parasites may happen in HIV patients with diarrhea and severely immune compromised; common transmission is anthropogenic and possibility of zoonotic transmission from felines and birds.

Acknowledgment

The authors thank to the British Council (DIID) for the funding through the DELPH-73 programme and Anthony Grimason for reviewing the manuscript. Special thanks to Prof. Huw V. Smith (deceased), who had been very supportive and encouraging to develop a standardized clinical parasitology laboratory in Jakarta.

References