ORIGINAL ARTICLE

Prevalence of *Cryptosporidium* spp. and *Blastocystis hominis* in faecal samples among diarrheic HIV patients in Medan, Indonesia

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ABSTRACT

Aim To investigate the prevalence of *Cryptosporidium* sp. and *B. hominis* among human immunodeficiency virus (HIV) positive patients in two different outpatient clinics, Haji Adam Malik General Hospital and Primary Care Centre of Padang Bulan, Medan, Indonesia, between two interval periods.

Method *Cryptosporidium* spp. microscopic examination, as well as Jones' medium for *B. hominis*, were conducted in the Parasitology Laboratory, enzyme-linked immunosorbent assay (ELISA) was done in the Multidisciplinary Laboratory, Faculty of Medicine, Universitas Sumatera Utara. This was a cross-sectional study, involving 54 diarrheic HIV positive patients (44 males, 10 females). The data were analysed by Spearmen rank correlation, interrater agreement, and 2 tests.

Results Infection rate for *Cryptosporidium* spp. and *B. hominis* was 24% (13 patients) and 9% (five patients), respectively. The prevalence of CD4 cell count below 200 cell/mm^3 was relatively high, 29.6% (16 patients). There was a significant relationship between cryptosporidiosis and CD4 cell count (p=0.01; OR 1.57; 95% CI 1.25-1.99). Microscopic examination was superior over ELISA, whose diagnostic value for sensitivity and specificity was 46.15% and 100.0%, respectively, and Kappa (K) coefficient of 0.56.

Conclusion The prevalence of cryptosporidiosis among HIV patients was still relatively high. CD4 count showed a significant relationship with *Cryptosporidium* spp. infection, but not with *Blastocystic hominis*. Microscopic examination is still the most reliable technique to diagnose the parasites in faecal samples.

Keywords: enzyme-linked immunosorbent assay, immunocompromised, intestinal parasite, Kinyoun-Gabbett, opportunistic protozoa

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INTRODUCTION

Cryptosporidium spp. has gained significant importance, particularly among the immunocompromised populations. In 1976, *Cryptosporidium* became more recognizable for its position causing gastroenteritis, followed by evidence of the infection associated with immunocompromised condition (1). Since then, *Cryptosporidium* spp. has emerged as one of the most common intestinal parasitic species worldwide. *Cryptosporidium* spp. is responsible for several outbreaks leading to higher mortality across the globe, particularly among the HIV population (2). This opportunistic intestinal protozoan has been co-infecting with HIV, especially those with low CD4 counts (3). *Cryptosporidium* spp. oocysts transmit mainly by the faecal-oral route; complementary transmission route was also included, such as direct contact from an infected individual or oocyst contaminated-food or water (4), although autoinfection within the same host is also possible (5). A spectrum of clinical manifestations developed in accordance with age, immune status, and nutritional status, and the agent's virulence and pathogenicity, whose factors highly depend on its special structure, rhoptries and micronemes (5). The symptoms are mostly self-limited, e.g. watery diarrhoea, abdominal cramps, light fever, and nausea and vomiting (6). A recent meta-analysis study has proven the association between antiretroviral therapy with the occurrence of cryptosporidiosis in HIV patients, thus proper management with chemoprophylaxis is recommended to reduce the risk of infection (3).

A stramenopile *B hominis* is the only organism in the group that could lead to human infection without any describable pathogenesis. There has been an extensive genetic variation of the organism causing elaborate investigation to understand its life and reproductive cycle (7). The advancement in molecular technique has recently unfolded the genetic diversity of the organism that finally reveals the detection and classification adequately. There are four main morphological forms of *Blastocystis* spp. in stools, i.e. vacuolar, granular, amoeboid, and cyst forms which proposed as infective stage (8). The prominent pathogenesis of *B. hominis* depends on its proteases activity and host intestinal microorganisms, all of which depend on organism subtypes (STs) that can be more supportive for *Blastocystis* sp. colonization (9). There are at least 17 distinct subtypes observed in several host species, humans and animals (10). The diagnosis of blastocystosis exposes a challenge for clinician, particularly since its appearance in wet mount is confused with *Cyclospora* spp., yeast, fat globules, helminths' ova, even sometimes leucocytes (11). It is also widely known that the infection commonly does not produce symptoms, yet symptomatic plus any presence of *B. hominis* from direct smear examination in patient stool could develop diagnosis and initiate medication (12).

Since both parasites cause non-specific symptoms and serve as opportunistic agents, clinicians do not always give enough consideration to these protozoans, even though a meta-analysis study has shown that *Cryprosporidium* spp. infection can lead to prolonged diarrhoea and devastating symptoms (3). This is partly caused by the lack of evidence on theses parasites, especially in Indonesia and other South East Asian countries.

The aim of the study was to investigate *Cryptosporidium* spp. and *B. hominis* infection rates among diarrheic HIV patients in two different outpatient clinics, as well as the detection rate of ELISA-based method compared to the microscopic examination using Kinyoun-Gabbett stain for *Cryptosporidium* spp.

PATIENTS AND METHODS

Patients and study design

This cross-sectional study was conducted in two different locations, i.e. HIV outpatient clinic in Haji Adam Malik General Hospital, and Padang Bulan Primary Care Centre, both located in the city of Medan, Indonesia. The data were collected within two interval periods, during June-August 2018 and June-September 2019, through a consecutive sampling process (all suitable samples were included in the study). All participants suffered from acute and chronic diarrhoea for several hours or days (three or more loose or liquid stools per day, based on the WHO criteria for diarrhoea) (13). Nevertheless, the study did not note this finding for further results/discussion.

Faecal samples were collected from each patient in two separate containers to accommodate unpreserved and formalin-preserved samples for further examination.

A total of 54 HIV positive patients had given their consent to enrol in the study and a brief oral explanation of the study protocol was conducted prior to the execution of the sampling process. Additionally, the data of accompanying variables, such as HIV positive status, age, gender, the latest CD4 cell count, and any presence of diarrhoea was noted in a short questionnaire.

The Ethical Committee for Medical Research of Faculty of Medicine, Universitas Sumatera Utara Indonesia has approved the study protocol (Reference number: 136/TGL/KEPK FK USU-RSUP HAM/2019).

Methods

Parasitological examination. Acid fast staining methods have been applied for the identification of *Cryptosporidium* spp (modified Ziehl-Neelsen and Kinyoun-Gabbett methods) (14,15). Kinyoun solution consisted of 4 grams of fuchsin alkaline, 8 grams of phenol, 5 mL of 95% alcohol, and 100 mL of sterilized water. Gabbett solution was made of 1 gram of methylene blue, 20 mL of 96% sulphuric acid, 30 mL of absolute alcohol, and 50 mL of sterilized water. The application of KG staining is like ZN staining, in which the sample is firstly applied with Kinyoun solution, followed by inundating the sample with Gabbett solution to maintain acid-fast stain nature of the oocyst under microscopic examination. An appearance of a 4-6 µL pinkish-red round shape was interpreted as *Cyrptosporidium* spp. oocysts.

B. hominis was revealed primarily using direct stain using Lugol's iodine and then positive *B. hominis* samples that underwent *in vitro* multiplication using Jones' culture medium (16). About 50 mg of unpreserved faecal samples were inoculated into Jones' medium and incubated at 37 °C; the growth was observed periodically for 24 hours. The culture would permit the appearance of reproductive and morphological stages of *B. hominis*. The procedure was also conducted to ensure the sample's positivity, both that the presence of at least one morphological form of *B. hominis* during direct microscopic examination was identified, and that was obtained from diarrheic patients with negative results. Furthermore, any positive appearance of *B. hominis* which was obtained from direct stain examination of cultured samples was declared positive.

Enzyme-linked immunosorbent assay (ELISA). The method was prepared to detect *Cryptosporidium parvum* antigen in faecal samples using the principle of microplate-based sandwich ELISA (Epitope Diagnostics, Inc., United States). The equipment for parasitic detection utilizes microtiter well, whose wall has been coated with highly purified antibody. It also consists of anti-Cryptosporidium tracer antibody, tracer antibody diluent, wash concentrate, HRP substrate, stop solution, and *Cryptosporidium* sp. antigen control (contained purified *Cryptosporidium* sp. oocysts). The interpretation was as follows: positive when patient's sample extinction was greater than the positive cut-off, and negative if patient's sample extinction was less than the negative cut-off.

Statistical analysis

The data were presented in univariate and bivariate modes, as well as further analysis to determine superiority between microscopic examination and ELISA for *Cryptosporidium* spp. detection. The test included sensitivity and specificity, as well as interrater agreement (Kappa, K) and Spearman correlation rank test.

RESULTS

A total of 54 HIV positive patients (44 males and 10 females) had voluntarily enrolled into the study and gave their faecal samples the following day. All participants (30 patients) admitted to the outpatient clinic in Haji Adam Malik General Hospital had low level of CD4 cell count and proved their continuation in receiving antiretroviral therapy (ART). One patient had infection with both *Cryptosporidium* spp. and *B. hominis* (with 10-day diarrhoea as the main symptom in addition to mild-moderate dehydration).

An exceptionally low level of CD4 cell count (below 200 cell/mm3) was evident among 10 hospitalized patients compared to six patients in the primary care centre (Table 1). Thirteen (24.1%) patients were positive for *Cryptosporidium* spp. (eight from the hospital and five from primary care centre) and five (9.3%) patients with blastocystosis (from the hospital and the primary care centre, three and two, respectively).

Significant relationship between low level of CD4 cell count and *Cryptosporidium* sp. infection (p=0.01; OR 1.57; 95% CI: 1.25-1.99) was

Table 1. Baseline characteristic of 54 HIV patients

Characteristic	N (%) of patients
Age (years)	
< 30	24 (44.4)
≥30	30 (55.6)
Gender	
Male	44 (81.5)
Female	10 (18.5)
CD4 cell count (cell/mm^3)	
>500	15 (27.8)
350-499	20 (37.0)
200-349	3 (5.6)
<200	16 (29.6)
***Cryptosporidium* spp**.	
Positive	13 (24.1)
Negative	41 (75.9)
Blastocystis hominis	
Positive	5 (9.3)
Negative	49 (90.7)

found, but not in the *B. hominis* infection, since only five samples were positive with the parasite (p=0.52; OR 0.54; 95% CI: 0.08-3.61). Spearman correlation rank test demonstrated significant findings between *Cryptosporidium sp.* and CD4 cell count (p=0.000; r=0.6) (Table 2).

Table 2. The infection prevalence and CD4 cell count among 54 HIV positive patients

Causative agent	N (%) of patients with CD4 cell count (cell/mm^3)			p	OR (95% CI)
	Low (<500)	Normal (≥500)	Total		
***Cryptosporidium* spp.**					
Positive	13 (100.0)	0 (0.0)	13 (100)	0.01	1.57 (1.25-1.99)
Negative	26 (63.4)	15 (36.6)	41 (100)		
Blastocystis hominis					
Positive	3 (60.0)	2 (40.0)	5 (100)	0.52	0.54 (0.08-3.61)
Negative	36 (73.5)	13 (26.5)	49 (100)		

The comparison of two diagnostic methods found that the microscopic examination was superior to ELISA. The greater number of findings (13 samples) was obtained using microscopic examination, while only six samples had positive results by using ELISA. Therefore, the diagnostic value of ELISA could be calculated, i.e. sensitivity 46.15%, specificity 100.0%, positive predictive value 100.0%, negative predictive value 85.42% with K=0.56 (Table 3).

Table 3. Comparison of diagnostic findings of Cryptosporidium sp. between Kinyoun-Gabbett (KG) stain and enzyme-linked immunosorbent assay (ELISA)

ELISA	No (%) of patients in KG stain			Weighted Kappa (95% CI)
	Positive	Negative	Total	
Positive	6	0	6 (11.1)	
Negative	7	41	48 (88.9)	0.56 (0.29- 0.83)
Total	13 (24.1)	41 (75.9)	54 (100.0)	

DISCUSSION

In this study, *Cryptosporidium* spp. infection rate among diarrheic HIV positive patients was 24% with the highest prevalence (84.6%) among the patients with very low CD4 cell count (<200 cells/mm^3), whereas the remaining positive samples (2 patients) was in 350-499 cells/mm3 group. Although all patients were admitted with diarrhoea, most patients did not demonstrate oocysts in microscopic examination. *B. hominis* infection rate was only 9.25% (five positive faecal samples). In Indonesia, a recent literature review has shown that *Cryptosporidium* spp. was found in 4-11% among diarrheic children, approximately 39% of among HIV/AIDS hospitalized patients with or without diarrhoea, and approximately 18.5% among HIV/AIDS hospitalized patients with tuberculosis (17). *Cryptosporidium* spp. has emerged as one of the most common enteropathogens causing gastroenteritis in adult patients, as well as one of the neglected tropical infectious diseases, which is due to the transformation in the environment, populations and demographics (18). Based on the Global Burden of Disease data in 2010, WHO has estimated more than 60,000,000 illnesses, 27,000 deaths, and 2.1 million disability adjusted life years (DALYs) lost were caused by *Cryptosporidium* spp. (19). Epidemiological trends of *Blastocystis* spp. infection demonstrate more frequently in human faecal samples and were found higher than *Giardia lamblia*, *Entamoeba* spp., and *Cryptosporidium* spp. in some regions (20). Studies in Ethiopia and Iran found the prevalence of *Blastocystis* infection ranging around 10.6-18.4% in HIV/AIDS patients (21-23), and significantly higher among those without ART (24). Most of those studies confirmed significant association between the infection and diarrhoea (22-24). The rate of infection was found much higher in Indonesia, which is 73.6% of 318 HIV patients (25).

The ability of *Cryptosporidium* spp. to produce symptoms fully depends on parasite characteristics and host factors, such as the immune competence and the frequency of exposure to parasite from infected individuals. All factors might result in varying symptoms, ranging from asymptomatic to life-threatening illness (26). One study reported significant association between cryptosporidiosis and each of CD4 count, diarrhoea duration,

abdominal cramps, fever, as well as nausea and vomiting (27). Dehydration can occur among vulnerable population, including children and immunocompromised patients, and associated with higher mortality rates (28). There are numerous reports on extraintestinal cryptosporidiosis, including in respiratory tract, pancreas, and biliary tract (29-32). In normal conditions, *Cryptosporidium* spp. will develop a membrane in the apical surface of intestinal epithelium that would not provoke systemic infection (5). Thus, it impairs the secretory and absorptive function of the gut epithelium recruiting inflammatory cells in localized tissue (33). Similarly, the unknown mechanism of *B. hominis* infection also leads to the confusion with other gastrointestinal infections. Diarrhoea, abdominal cramps, nausea vomiting, or other non-specific symptoms could also accompany the presence of *B. hominis* in fecal samples (34).

Immune response is problematic for patients with impaired cellular immune function, in which the major reaction will involve T-cell immunity that weakens the eradication process of the infection (35). It is obvious that the self-limiting nature of cryptosporidiosis will vanquish and become more disastrous in immunosuppressed population (5). There were no reports of symptoms and patient's quality of life in this study, but it confirmed the relationship of lower level of CD4 cell count with higher incidence of cryptosporidiosis. In contrast, *B. hominis* infection has no significant relationship with CD4 cell count, yet descriptively it was evident that the infection rate of *B. hominis* was higher among lower CD4 group.

The pathogenesis of infection remained elusive for several decades, but several proposed pathogeneses have been reported in many studies, which is related to cysteine proteases and other proteolytic enzyme secretion (36,37). The production of lysates could induce dramatic changes in cytoskeleton that finally promotes apoptosis in epithelial cells, while cysteine protease will be encoded by *B. hominis* leading to IL-8 production from intestine epithelial cells (36).

Our study also demonstrated low sensitivity of ELISA compared to microscopic examination as a golden standard through KG stain. There were also high false negative samples among cryptosporidiosis patients, which profoundly presented in microscopic examination. The enzyme assay has been evaluated for decades with varying degree of accuracy, from low sensitivity (40.9%) and higher specificity (78.9%) using animal faecal samples, which was positive for *Cryptosporidium* spp. (38), whilst some other reports found excellent diagnostic value of ELISA-based method, whose specificity and sensitivity were ranging around 92.25-96% and 87.38-100% respectively (39-43). The method also showed best performance compared to other techniques in testing *B. hominis*, including direct wet mount, trichrome stain, and in vitro culture (44). Notwithstanding its fair/substantial agreement of Kappa value (0.56), our study was not successful to prove ELISA superiority to microscopic examination. There are several aspects affecting ELISA results, mainly the technical aspects of the equipment and experts who conduct the examination relating to the multiple steps of sandwich ELISA (45). The disadvantage of direct microscopic examination is greater affected by the operator who conducts the procedure, particularly relating to the presence of oocyst or other morphological form of organism (46). However, the direct microscopic examination is significantly cheaper and more affordable when conducted properly.

In conclusion, *Cryptosporidium* spp. infection should be regarded in managing immunocompromised patients, especially those with low CD4 levels. In addition, microscopic examination remains the gold standard for *Cryptosporidium* spp., since it creates less technical problems relating to multistep procedure, particularly for low-cost laboratories in developing countries.

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TRANSPARENCY DECLARATION

Conflict of interest: None to declare.

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