RESEARCH ARTICLE

Prevalence of Intestinal Parasitic Infections and Genotypic Distribution with Risk Factors of *Enterocytozoon bieneusi* in HIV Patients of Maesai Hospital, Thailand

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Abstract

Co-infections of HIV and intestinal parasites including *Enterocytozoon bieneusi* (*E. bieneusi*) are a common cause of gastrointestinal syndrome in HIV-infected people. Our study was designed to detect infection rates of intestinal parasites and the genotypic distribution of *E. bieneusi*, as well as probable risk factors for infection in HIV patients at Maesai hospital, Chiang Rai, Thailand. Two hundred and twenty-four stool samples from enrolled HIV-infected participants were collected and examined for parasitic infections, using microscopy and polymerase chain reaction techniques. CD4 status as well as the demographic data of HIV-infected participants was also collected and analyzed. Intestinal parasites including *E. bieneusi* were detected in 4.02 % of all participants. The highest intestinal parasitic infection rate was *E. bieneusi* (2.23 %) followed by *Strongyloides stercoralis* (1.34 %), *Opisthorchis viverrini* (0.89 %) and *Giardia intestinalis* (0.45 %). Intestinal parasitic infection rate of participants with CD4 count $\leq$ 200 cells/mm^3 was significantly higher than that of participants with CD4 counts > 200 cells/mm^3 (12.50 % vs 2.63 %, p = 0.027). Correspondingly, the infection rate of *E. bieneusi* was significantly higher in participants with CD4 count $\leq$ 200 cells/mm^3 than in participants with CD4 count > 200 cells/mm^3 (9.38 % vs 1.05 %, p = 0.022). Two genotypes of *E. bieneusi*, including D (n = 3) and SH8 (n = 2), were identified from 5 participants. Both of the identified genotypes were likely a zoonotic transmission. Human infection by *E. bieneusi* genotype SH8 was a discovery for the first time in Thailand. However, no intestinal coccidian infections were diagnosed. The low numbers of intestinal parasitic infections of this study were probably due to wide availability of antiretroviral therapy, improved health sanitation, as well as ease of access to antiparasitic medication in HIV-infected people.

Keywords: Intestinal parasites, Microsporidia, *E. bieneusi*, HIV, CD4, Thailand

Introduction

Human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) remains an ongoing health problem. UNAIDS reported that Thailand has the highest number of people living with HIV and AIDS-related deaths in the South East Asia region [1]. HIV causes a variety of opportunistic illnesses in infected people, with enteric infections by microorganism being one of the most important etiological factors related to morbidity and mortality in HIV/AIDS patients [2,3]. Depletion of CD4 cells in HIV patients resulting in the progressive decline of immunological responses makes susceptible to either opportunistic or non-opportunistic intestinal parasites [3-5].

Infection of opportunistic intestinal parasites in HIV patients is associated with diarrhea, especially coccidian infection with low CD4 cell count [6-8]. Microsporidia are other important opportunistic intestinal parasites. They show widespread distribution in HIV patients, especially in those with a CD4 cell count of less than 200 cells/mm^3 [9,10]. Of the 17 species of microsporidia that infect humans, *E. bieneusi* is the major etiologic agent [11-13]. Infection of *E. bieneusi* has the pathogenic potential of gastrointestinal tract resulting in chronic diarrhea, intestinal malabsorption, and debilitation in HIV-infected people [14]. Genotypic analysis of *E. bieneusi* can be used to predict their mode of transmission [15-20]. In developing countries, including Thailand, the transmission of *E. bieneusi* occurs following either anthroponotic or zoonotic transmissions [12,14,19]. Over the past decade there have not been many reports on the prevalence of intestinal parasites as well as molecular epidemiological studies, and data available on the transmission

of *E. bieneusi* in HIV patients in Thailand. Chiang Rai province, especially Maesai District, is one of the highest prevalence of HIV infection area with a multiethnic social environment in Thailand [21].

Therefore, we aimed to determine the prevalence of either opportunistic or non-opportunistic intestinal parasites, including *E. bieneusi* genotypic distribution and possible risk factors of *E. bieneusi* infection in HIV patients at Maesai hospital, Chiang Rai, Thailand.

Materials and methods

Ethics statement

The protocol of this study was reviewed and approved by the Ethical Committee of Mae Fah Luang University (reference no. REH60101) following the principles expressed in the Declaration of Helsinki. Participating individuals were included in the study on a voluntary basis. All the participants were clearly informed of the purpose and their right in this study. Furthermore, the participants' personal information was protected confidentially. All parasitic infections diagnosed were treated using a standard treatment protocol.

Study sites and population

During November 2017 to March 2018, 224 HIV patients at antiretroviral therapy (ART) clinic, Maesai hospital were recruited to participate in the study. This hospital is a secondary hospital with a capacity of 90 beds and is located in the northernmost part of Thailand. Maesai hospital provides health services to various ethnic groups due to its location close to the Thailand-Myanmar border. Participating individuals were over 20 years of age and follow-up HIV cases. Information of each participant, including age, nationality, type of drinking water and type of domestic pets was collected. Single stool sample from each participant was collected and analyzed. The recent CD4 data of each participant were collected from the hospital information system in which the data were assessed regarding to intestinal parasitic infection.

Intestinal parasite analysis by microscopy

About 1 g of stool was examined using the formalin-ether centrifugal sedimentation technique [22]. A part of the stool sediment was directly smeared and then examined microscopically for protozoa and helminths diagnosis. Another part of the sediment, about 40 μl, was used to perform modified acid-fast staining [23], for coccidian diagnosis. Briefly, the thin smear was prepared and allowed to air dry. The slide was fixed with methanol for 3 min and stained with carbol fuchsin for 15 min. The slide was rinsed with tap water and decolorized with 3 % hydrochloric acid in ethanol for 15 s. The slide was then rinsed in tap water and counterstained with 1 % methylene blue for 1 min. The stained slide was observed microscopically.

Detection of *E. bieneusi* by polymerase chain reaction (PCR)

Each fresh stool of the patients was kept at −80 °C to preserve for the DNA extraction process. About 200 mg of the frozen stool was required for DNA extraction using QIAamp DNA stool mini kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The extracted DNA of each sample was kept frozen at −20 °C until used for detection of *E. bieneusi* DNA by PCR. A PCR was done to amplify the internal transcribed spacer (ITS) region of the rRNA gene of *E. bieneusi* [24,25]. In brief, PCR was conducted in a 25 μl reaction, containing 1X PCR buffer, 2.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.16 μM of each primer MSP-3 (5'-GGA ATT CAC ACC GCC CGT C(A/G) (C/T) TAT-3') and MSP-4B (5'-CCA AGC TTA TGC TTA AGT CCA GGG AG-3') and 1 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA). PCR condition was run with 50 cycles of 92 °C for 60 s and 58 °C for 60 s, followed by 72 °C for 90 s. The amplified products were run on a 1.5 % agarose gel electrophoresis. The gels were stained with ethidium bromide and visualized under UV light using gel documentation system (Syngene, cambridge, UK).

DNA sequencing and analyzing of *E. bieneusi*

All positive PCR products of *E. bieneusi* were delivered to U2Bio Company Limited (Thailand) for DNA purification and sequencing. Briefly, a PCR product, 508-bp fragment, was purified by agarose gel extraction, and this was followed by sequencing on an ABI Prism 3037 XL DNA analyzer (Applied Biosystems, Foster City, CA, USA) using the PCR primers. Two-directional sequencing was done to ensure the accuracy of DNA sequences. To identify *E. bieneusi* genotypes, the Basic Local Alignment Search Tool (BLAST) was used to compare homology of sequenced ITS (243 bp) of PCR products to the ITS sequence in the GenBank database.

The evolutionary relationships among various genotypes of *E. bieneusi* were evaluated by phylogenetic tree analysis using ITS sequences in this present study along with some ITS sequences from the GenBank database. The neighbor-joining tree was constructed using Kimura 2-parameters model and bootstrap analysis with 1,000 replicates by the program MEGA 10 [26].

Statistical analysis

Data were analyzed using SPSS Statistics 21.0 (IBM, USA). The chi-square test was used to determine the distribution of the patients with intestinal parasite infection, according to their CD4 status (2 groups: ≤ 200 cells/mm^3 and > 200 cells/mm^3). Where appropriate the Fisher exact test was utilized to determine *p*-value. $p \leq 0.05$ were considered as of statistical significance.

Results and discussion

Study population and intestinal parasitic infections

A total of 224 HIV patients were examined for intestinal parasitic infections, 91 (40.6 %) were males and 133 (59.4 %) were females, including 32 patients with CD4 counts less than or equal to 200 cells/mm^3, and 190 patients with CD4 count greater than 200 cells/mm^3 (the CD4 count was not available for 2 patients) (**Table 1**). The median age was 45 years (age range: 20 - 77). There were 6 ethnic minority groups represented in the HIV patients recruited i.e., Thai (57.1 %), Shan (19.1 %), Tai Lu (13.4 %), Burmese (5.6 %), Akha (0.8 %) and Lua (0.4 %) along with a not identified ethnic group (3.6 %) (**Table 1**). The parasite species diagnosed and the corresponding CD4 counts are shown in **Table 2**. According to examination of these intestinal parasitic infections, at least 1 parasite species was identified in 9 patients (4.02 %) and 2 parasite species were identified in 2 patients (0.89 %). For 2 parasite species infection, both of the patients were infected with *Strongyloides stercoralis* (*S. stercoralis*) and *Opisthorchis viverrini* (*O. viverrini*). Males had significantly higher intestinal parasitic infection rates than females ($p = 0.033$). There were no opportunistic coccidian infections observed in this study. The prevalence of intestinal parasitic infections of the patients with CD4 count less than or equal to 200 cells/mm^3 (12.50 %) was significantly higher than those of the patients with CD4 count greater than 200 cells/mm^3 (2.63 %), with a *p*-value = 0.027. *E. bieneusi* had the highest prevalence of 2.23 %, followed by *S. stercoralis* (1.34 %), *O. viverrini* (0.89 %) and *Giardia intestinalis* (*G. intestinalis*) (0.45 %). The prevalence of *E. bieneusi* was significantly higher in the patients with CD4 count less than or equal to 200 cells/mm^3 than in those with CD4 count greater than 200 cells/mm^3 (9.38 % vs 1.05 %, $p = 0.022$). For detection of *E. bieneusi* by PCR, all the positive samples showed 508 bp of PCR product of rRNA gene which covered the entire ITS region (**Figure 1**).

Table 1 Demography and CD4 status of HIV patients accessed ART clinic of Maesai hospital (n = 224).

Demographic features	No. of male	No. of female	Total number (%)
Age group (20 - 77 years)			
< 45 years	39	70	109 (48.7)
≥ 45 years	52	63	115 (51.3)
Ethnicity			
Thai	63	65	128 (57.1)
Shan	16	27	43 (19.1)
Tai Lu	6	24	30 (13.4)
Burmese	6	6	12 (5.6)
Akha	0	2	2 (0.8)
Lua	0	1	1 (0.4)
Not identified ethnics	0	8	8 (3.6)
CD4 count (cells/mm^3)			
≤ 200	22	10	32 (14.3)
> 200	69	121	190 (84.8)
No data	-	2	2 (0.9)

Table 2 Intestinal parasitic infections in HIV patients by CD4 status.

Parasites	Total, n = 224 Positive no. (%)	CD4 ≤ 200 cells/mm³, n = 32 Positive no. (%)	CD4 > 200 cells/mm³, n = 190 Positive no. (%)	*p*-value
Any parasite	9 (4.02)	4 (12.50)	5 (2.63)	0.027*
2 parasite species	2 (0.89)	0 (0)	2 (1.05)	1.000
Strongyloides stercoralis	3 (1.34)	1 (3.12)	2 (1.05)	0.375
Opisthorchis viverrini	2 (0.89)	0 (0)	2 (1.05)	1.000
Giardia intestinalis	1 (0.45)	0 (0)	1 (0.53)	1.000
Enterocytozoon bieneusi	5 (2.23)	3 (9.38)	2 (1.05)	0.022*

*p-v*alue of Fisher exact test.
*$p < 0.05$ was considered statistically significant
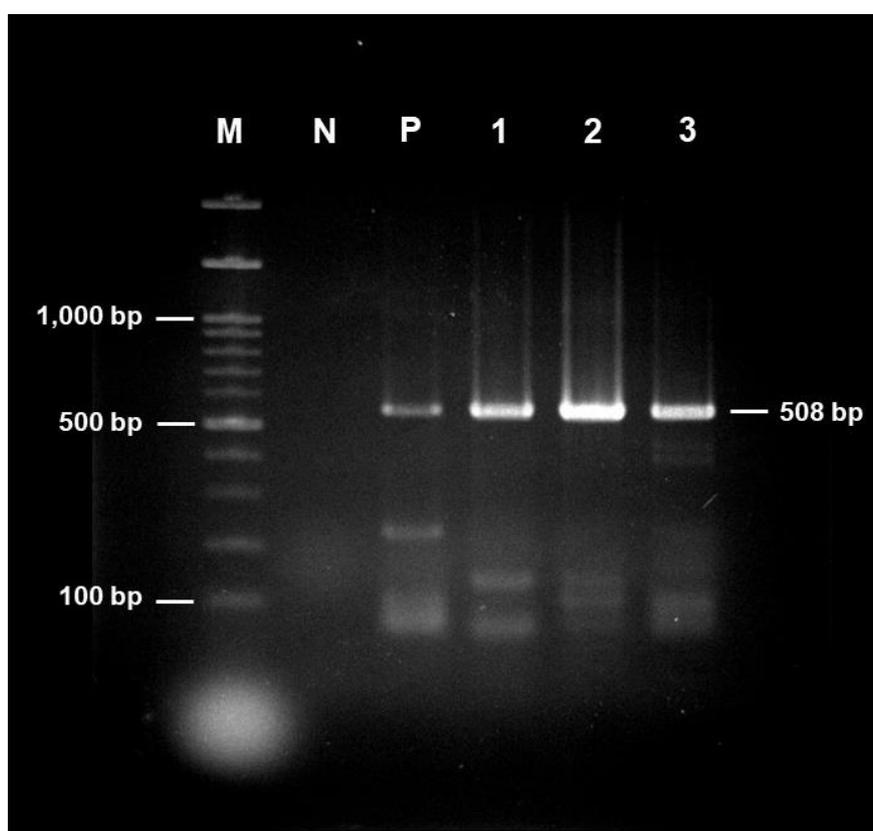

Figure 1 Agarose gel electrophoresis of PCR products of *E. bieneusi* using the MSP3-MSP4B primer pair specific to the small subunit rRNA gene. Lane M: Molecular markers of 100-bp DNA ladder, lane N: Negative control, lane P: Positive control, lanes 1 to 3: Positive samples for *E. bieneusi*.

The prevalence rates of non-opportunistic intestinal parasites including *S. stercoralis* (1.34 %), *O. viverrini* (0.89 %) and *G. intestinalis* (0.45 %) in this study were lower than those of *S. stercoralis* (3.33-26.2 %), *O. viverrini* (1.1 - 19.2 %) and *G. intestinalis* (1.3 - 35.2 %) among HIV patients of previous studies in various regions of Thailand [27-29]. Improvements in sanitation, personal cleanliness, water supply quality, basic health education, and antiparasitic drugs' widespread could explain the observed variation. In HIV/AIDS patients, intestinal opportunistic parasite infections remain a major public health concern, particularly in those with a CD4 count less than 200 cells/mm³ [10,30]. Previous studies on HIV patients found that the prevalence of the most common coccidia, *Cryptosporidium* spp., ranged from 3.3-30.0 % in Thailand [27,29,31,32], and 6.6 % in Lao PDR [33]. Even though cryptosporidiosis has been linked to HIV infection and contaminated water consumption, no *Cryptosporidium* was discovered in HIV

patients in this study. *Cryptosporidium* is a waterborne pathogen which is used to be an indicator of drinking water quality [34]. In remote areas of Thailand, the main sources of drinking water and other purposes for consumption are mountain water and/or local water supply [35]. Our study site, Maesai district, Chiang Rai province, is located in the far north of Thailand, near the Myanmar border, where the provincial waterworks authority does not deliver water. Although some residents in this area consumed groundwater and local water supply, they improved the water quality before drinking it, for example, by filtering, boiling, and also purchasing bottled water. This could mean that living standards have improved in terms of sanitary environmental conditions, reducing the risk of *Cryptosporidium* and other waterborne infections.

Since 1985 microsporidia, particularly *E. bieneusi*, has often been detected in AIDS patients, producing chronic diarrhea and wasting syndrome [17]. In this study, *E. bieneusi* had the highest prevalence among the various intestinal parasites (**Table 2**). Furthermore, the patients with CD4 counts less than or equal to 200 cells/mm^3 had a prevalence rate of *E. bieneusi* (9.38 %) which was statistically significantly higher than the patients with CD4 counts greater than 200 cells/mm^3 (1.05 %). According to several studies, the prevalence rates of microsporidiosis in HIV-infected people were 1.67 % in Bangkok, Thailand [29], 81.2 % in Lopburi, Thailand [32], 5.6 % in a Thai AIDS care center [31], and 2.9 % in Lao PDR [33]. The variation in prevalence rates could be attributed to the use of different methodologies, study populations and time periods. Most of the previous studies used microscopic techniques which detected all species of microsporidia with lower sensitivity [29,32,33], while we used PCR to detect only *E. bieneusi* with higher sensitivity. As a result of the differences in diagnostic methods, comparing prevalence rates is problematic. Although, *E. bieneusi* infection was associated with the presence of chronic diarrhea in HIV/AIDS patients, we found that all 5 positives of *E. bieneusi* did not have diarrhea. Similarly, HIV-infected children of Thai orphanages, with *E. bieneusi* infection (2.6 % prevalence rate) also had no diarrhea [36]. However, in developing countries, *E. bieneusi* prevalence rates were reported 2 - 51 % of HIV-infected adults with diarrhea and 4.6 % of HIV-infected adults without diarrhea [14].

Our study showed low prevalence of intestinal parasites in HIV patients who on ART. According to previous studies, on-ART HIV patients showed that the prevalence of intestinal parasitic infections was significantly lower than naïve or pre-ART HIV patients [37-40]. ART appears to be efficient in lowering the HIV viral load, with a quantitative and qualitative improvement in the CD4 status, leading to a significant reduction in opportunistic infections including parasitic infections [41]. Due to the Thailand universal coverage scheme, all Thai citizens have free access health care services including ART [42]. Therefore, HIV patients now have more access to ART resulting in better health conditions.

Genotypic distribution of *E. bieneusi*

In this study, 2 known genotypes of *E. bieneusi* were identified in 5 patients, composing 3 of genotype D and 2 of genotype SH8 (**Table 3**). Each ITS sequence of the identified genotypes was deposited representatively in the GenBank database including MZ172788 as a genotype D and MZ172787 as a genotype SH8. The sequences of ITS in this study and those in the GenBank database showed 100 % homology. These 2 identified genotypes were phylogenetically indicated into group 1 which related to human pathogenic genotypes. Genotype D was assigned to subgroup 1a whereas genotype SH8 was assigned to subgroup 1e (**Figure 2**). Group 1 is the largest genetic group which has a wide range of hosts and geographical regions. Within group 1, genotype D is the most commonly found in humans, as well as domestic and wild animals, while genotype SH8 has been predominantly found in domestic pigs and wild boars [16,43]. In Thailand, genotype D has been found in HIV patients, water sources, and a wide range of animals, including pigs and cats, implying the possibility of waterborne and zoonotic transmissions [16,44,45]. Genotype SH8 has been reported in humans, pigs and wild boars in China [16,43]. To our knowledge, this is a first-time identification of genotype SH8 in humans in Thailand. Two of the five *E. bieneusi*-infected cases in our study raised animals such as poultry, dogs and cats, and both cases had a CD4 count of less than or equal to 200 cells/mm^3 (**Table 3**), suggesting that these could be infection risk factors. Furthermore, inadequate hygienic sanitation, such as non-boiled drinking water, may increase the risk of *E. bieneusi* infection [46]. However, because all positive cases consumed commercial bottled water, there was no link between *E. bieneusi* infection and water as a risk factor in our study. Similarly, a research study in Myanmar found no significant differences in *E. bieneusi* infection rates when consuming boiled or non-boiled water [47]. Nevertheless, *E. bieneusi* genotypes within genetic group 1 should be considered a public health concern because of their widespread occurrence in a variety of hosts [16].

Table 3 *E. bieneusi* genotypes in HIV patients (5 positive cases of total 224 patients).

Case No.	*E. bieneusi* genotype	Sex	Ethnic	CD4 count (cells/mm^3)	Type of drinking water	Animal in household
1	D	male	Thai	134		chickens
2	D	male	Thai	737		none
3	D	female	Shan	449	Bottled water	none
4	SH8	male	Shan	183		dogs and cats
5	SH8	female	Burmese	125		none
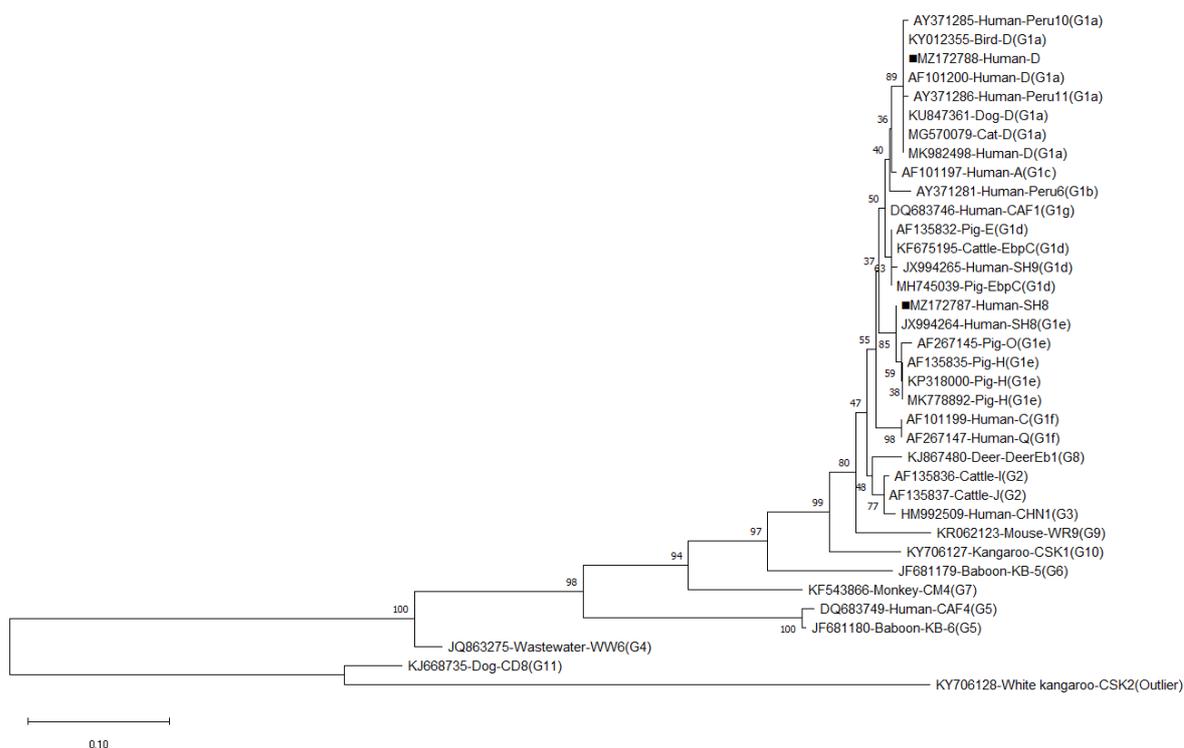
Figure 2 Phylogenetic analyses of *E. bieneusi* genotypes identified in this study and in other reports. The phylogeny was inferred with a neighbor-joining analysis of the ITS sequences based on distances calculated with the Kimura 2-parameter model. The numbers on the branches are percentage bootstrapping values of 1,000 replicates. Each sequence is indicated by its GenBank accession number, host origin and genotype designation, along with phylogenetic group, as shown in parenthesis. Genotypes identified in this study were indicated by ■.

Limitations of study

We collected single stool sample from each HIV patients. Therefore, intestinal parasite burden in this study might have been underestimated. We also lacked intestinal parasite burden in healthy population of the study area as a control group which may reflect naturally low prevalence of intestinal parasites. In addition, the limited sample size and the specific geographical and socio economical setting of our study, may require a larger and nationwide study to provide stronger results. Nevertheless, despite these limitations we believe in the useful information of our study particularly in terms of screening at least for those treatable parasites and concerning of intestinal parasitic health implications.

Conclusions

In this study, the low prevalence of both opportunistic and non-opportunistic intestinal parasitic infections in HIV patients of Maesai Hospital was probably because of ART accessibility, health sanitation improvement, and ease of access to antiparasitic drugs with no prescription. Genotypes D and SH8 of *E. bieneusi* were identified in which genotype SH8 found in the patients was the first to be reported in Thailand. Risk factors of infection of *E. bieneusi* genotypes D and SH8 are likely to be low number of CD4 cells and interaction with animals. Further investigation in animals is needed to address further epidemiological characteristics of *E. bieneusi* infection.

Acknowledgements

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