mM EDTA, pH 8.0) and stored at -20°C until used.

Polymerase chain reaction. The DNA fragment spanning the SSU rRNA, ITS-1, 5.8S rRNA and ITS-2 regions of *I. belli* was amplified by nested polymerase chain reaction (PCR) using primers whose sequences were derived from the 5' portion of the SSU rRNA gene of *I. belli* isolate CI1 (accession number U94787) and the 5' portion of the 28S rRNA gene of *I. felis* (accession number U85705). Sequences of the outer pair of primers were Iso-18SF0, 5'-CTGGTTGATCCTGCCAGTA-3' and Iso-28SR0, 5'-AAGGCTCAATCAAGAACCTCCG-3', and the inner pair, Iso-18SF1, 5'-GATCCTGCCAGTAGTCAT-3' and Iso-28SR1, 5'-TGAAGCTAATCCCTCTTCC-3'. We used the same thermal cycling profiles for both primary and secondary PCR: denaturation at 94°C, 40 s; annealing at 64°C, 40 s; extension at 74°C, 5 min, and 35 cycles of amplification. DNA amplification was performed by using a GeneAmp 9700 PCR thermal cycler (Applied Biosystems, Foster City, CA). To minimize the error introduced in the sequences during PCR amplification, we used ExTaq DNA polymerase that possesses efficient 5'→3' exonuclease activity to increase fidelity and no strand displacement (Takara, Japan). The size of PCR product was examined by electrophoresis in a 1% agarose gel and visualized under a UV transilluminator (Mupid Scope WD, Japan). The PCR product was purified by using QIAquick PCR purification kit (QIAGEN, Germany).

Subcloning. The GeneJET^{TM} PCR Cloning Kit was used for ligating the purified PCR product to the plasmid vector pJET1/blunt (Fermentas, Canada). After incubation at 15°C for 6 hours, the reaction mixture was precipitated, redissolved in 10 μl of double distilled water and transformed into *Escherichia coli* strain JM107 by electroporation using an *E.coli* pulser apparatus (BioRad, USA). Recombinant DNA from positive clones was prepared by QIAGEN plasmid mini kit (QIAGEN, Germany).

DNA sequencing. DNA sequences were determined directly from both PCR-purified templates and plasmid subclones. Sequencing analysis was performed from both directions for each template using the Big Dye Terminator v3.1 Cycle Sequencing Kit on an ABI310 Genetic Analyzer (Applied Biosystems, USA). Overlapping sequences were obtained by using sequencing primers (available upon request). Whenever singleton substitution occurred, sequence was re-determined using PCR products from two independent amplifications from the same DNA template.

Data analysis. Sequences were aligned according to their primary and secondary structures

by the CLUSTAL X and the MARNA programs, respectively.[13,14] Phylogenetic construction was performed by the neighbor-joining method using Kimura-2-parameter and 1,000 bootstrap iterations as implemented in the MEGA version 3.1 program.[15] To analyze the evolutionary relationship, homologous sequences of other coccidian parasites available in the GenBank database were incorporated for comparison: *I. belli* (AF106935), *I. ohioensis* (AF029303), *I. suis (U97523), I. orlovi* (AY365026), *I. felis* (L76471), *Toxoplasma gondii* (M97703 and L49390), *Cryptosporidium parvum* (AF093490 and AF015773), *Cyclospora cayetanensis* (U40261 and AF301391), *Neospora caninum* (U16159 and L49389), *Hammondia heydorni* (AF317282), *H. hammondi* (AF096498), *Eimeria tenella* (U40264) and *E. papillata* (AY779501). New nucleotide sequence data obtained in this study have been deposited in the GenBank databases under the accession numbers DQ060658-DQ060683.

Results:

Patient profiles. Between January 2002 and December 2004, stool samples from 39,510 individuals were submitted to parasitology laboratory of King Chulalongkorn Memorial Hospital in Bangkok for examination of enteric parasites. Of these, 497 were from confirmed HIV/AIDS patients. Isosporiasis was identified in 38 patients by the presence of *I. belli* oocysts in their stool samples: 12 found by direct wet smear method (31.6%), 37 by formalin-ethylacetate sedimentation (97.4%) and 36 by modified kinyoun acid fast stain (94.7%). Isosporiasis patients comprised 35 Thai from diverse regions of the country and 3 recent immigrants from Cambodia, Laos and Pakistan.

Of all *I. belli*–infected patients, 30 were HIV-positives, 3 received prolonged corticosteroid therapy for systemic lupus erythematosus or idiopathic thrombocytopenic purpura, and 5 immunocompetent individuals. More than half of isosporiasis patients were 30-39 years old and the ratio of male to female 1.38:1. Laboratory tests showed relative eosinophilia (>4%) in half of HIV-positive cases and all immunocompetent patients but none in the patients under prolonged corticosteroid treatment (Table 1). The absolute CD4+ lymphocyte counts in HIV-positive cases were 8-484 cells/μl (mean±S.D.=67.5±57.4 cells/μl). Among HIV-infected cases, concurrent infections with one or more of the following pathogens occurred in 9 patients that included herpes simplex, herpes zoster, *Histoplasma capsulatum*, *Mycobacterium tuberculosis*, *Salmonella* group B and *Strongyloides stercoralis*.

All HIV-infected individuals developed watery diarrhea ranging from 1 week to 1 year (mean±S.D.=3.3 ±3.4 months) prior to attending King Chulalongkorn Memorial Hospital while 2 patients who received corticosteroids presented with chronic watery diarrhea and the other without symptom. The clinical profiles of isosporiasis in immunocompetent patients were asymptomatic, chronic watery diarrhea, and dyspepsia without diarrhea, occurring in 2, 2 and 1 case, respectively. Diarrhea and associated gastrointestinal symptoms resolved within a few days after initiation of treatment with combination of trimethoprim and sulfamethoxazole. During 2 months of follow up, relapse occurred once each in 2 HIV-infected and 1 immunocompetent patient that could be controlled by long term prophylaxis with trimethoprim and sulfamethoxazole.

Oocyst morphometry. The oocyst dimension of each isolate varied from 17 to 37 μm in length (mean±S.D.=28.3±3.0 μm), 8 to 21 μm (mean±S.D.=13.5±1.9 μm) in width and the mean±S.D. shape index (length by width) was 2.1±0.31 (range 3 to 3.3). Although the oocysts exhibited shape and size variations both within and between isolates, the shape indices of all oocysts observed in this study were consistent with that of *I. belli*, being more than 1.2, which was distinct from those for other species of *Isospora* infecting nonhuman mammals (<1.2).[1]

Sporulation of oocysts. We were able to follow oocyst maturation using fresh watery stool samples from 2 HIV-infected patients and one immunocompetent subject who had not yet taken anti-coccidial drugs. In total, 100 oocysts were observed for each isolate. The average number of oocysts of these samples that underwent complete formation of sporozoites was 27 (range=20-33). The duration for an oocyst to transform into 2 sporocysts, each of which contained 4 sporozoites, ranged from 24 hours to 10 days (mean±S.D.=3.9±3.4 days)(n=66). Interestingly, *Caryospora*-like oocyst, characterized by oocyst containing one sporocyst which enclosed 8 sporozoites, appeared on day 5-14 after incubation. Although this type of oocyst could be found in all 3 isolates, they occurred at low percentage (~5%)(Figure 1). The dimension of *Caryospora*-like oocysts did not differ significantly from those having bisporocyst (data not shown).

The SSU rRNA, ITS-1, 5.8S rRNA and ITS-2 sequences. The DNA fragment generated from the secondary PCR encompassing the SSU rRNA, ITS-1, 5.8S rRNA and ITS-2 genes contained 3,049 base pairs. All isolates yielded single PCR fragments of identical size (data not shown). Of these, we determined the sequences of 26 isolates from HIV-infected patients (n=23), symptomatic immunocompetent individuals (n=2) and an asymptomatic immunocompetent subject. We did not find any remarkable preponderance toward purine or pyrimidine composition

in the region analyzed (%GC = 47.1). Sequence of the SSU rRNA gene, spanning 1,778 base pairs, of 25 isolates examined were identical with those of strains CI1 and CJLPHD2 (accession numbers U94787 and AF441289) but differed from the isolate reported by Franzen et al (accession number AF106935) at A679T and A682C.[16] Meanwhile, 3 additional nucleotide substitutions occurred at T583C, C638A and G1240T in the isolate from an immunocompetent patient who suffered from multiple relapses.

The 5.8S rRNA, ITS-1 and ITS-2 regions contained 598, 158 and 404 base pairs, respectively. Both the 5.8S rRNA and ITS-2 sequences of *I. belli* exhibited perfect sequence identity among all isolates examined. Likewise, sequences of the ITS-1 were highly conserved with the only exception of one nucleotide substitution at position 528 with an A to G exchange (position according to the ITS-1 region). However, there was no association between this single nucleotide exchange and clinical severity of the infected individuals. Meanwhile, comparison of the 5.8S rRNA of *I. belli* with those of other coccidian parasites revealed high similarity of sequences (Figure 2). On the contrary, the ITS-1 and ITS-2 regions exhibited extensive sequence diversity.

Phylogenetic analysis. The neighbor-joining tree derived from the SSU rRNA sequences confirmed that all isolates of *I. belli* in this study were clustered together, consistent with a single species (Figure 3). Further analysis using the available complete sequences of the SSU rRNA gene of the genus *Toxoplasma*, *Cryptosporidium*, *Cyclospora*, *Isospora*, *Neospora*, *Eimeria* and *Hammondia,* that were aligned according to their secondary structure, has shown that *I. belli* was more related to *Toxoplasma*, *Neospora* and *Hammondia* than to *Cyclospora*, *Eimeria* and *Cryptosporidium*. Consistently, an identical topology of phylogenetic tree to that inferred from the SSU rRNA sequences was reaffirmed when the ITS-2 sequences were used for comparison (Figure 4).

Discussions:

We searched for isosporiasis in a large number of patients who submitted their stool samples to King Chulalongkorn Memorial Hospital during 3 year period. The overall prevalence of *I. belli*-positive samples was around 0.1% based on the total samples examined. The prevalence of isosporiasis among HIV/AIDS patients in this study was 6.0% and decreased to 0.02% in non-HIV-infected cases. However, similar cross-sectional studies in Germany, Haiti and Venezuela

showed a higher prevalence, ranging from 14 to 48%.[4,6,17] The low prevalence of isosporiasis in this study suggested that *I. belli* might not commonly circulate in Thai population, resulting in a low prevalence of isosporiasis in HIV/AIDS patients in this country. Nevertheless, the majority of isosporiasis patients in Thailand occurred in those who had immunosuppression, comprising 86.8% of all positive cases.

Despite a rapid improvement of symptoms after oral trimethoprim and sulfamethoxazole treatment, recurrent isosporiasis occurred in 3 HIV/AIDS patients (~8%) within 2 months after initiation of treatment. Importantly, about half of Haitian patients who received the same or similar treatment had relapse.[5] It could be that the duration and frequency of follow up after treatment contributed to the difference in the observed relapsing episodes from each study. However, it seems that continuing anti-coccidial prophylaxis will be warranted in certain isosporiasis cases.

The life cycle of *I. belli* depends solely on monoxenous development in a human host without known natural animal reservoir or paratenic host. Both schizogonic and sporogonic developments take place intracellularly in epithelium of small intestine. However, the stages of oocysts that are newly passed in feces mostly contain a single sporont, but oocyst with 2 sporoblasts can be occasionally encountered. To observe the oocyst development ex vivo, we used fresh stool samples without preservative, such as potassium dichromate, in order to envisage the fate of oocyst development when excreted to environment. The duration for complete sporulation of *I. belli* described herein is rather variable. Although we used different experimental conditions from other studies, the earliest appearance of fully sporulated oocyst is similar, i.e. within 24 hours after passage from the host.[2,3]

It is well recognized that mature oocysts of *I. belli* produce 2 sporocysts, each with 4 sporozoites. Interestingly, a small percentage around less than 2% of oocysts that underwent sporogonic development contained 8 sporozoites in a single sporocyst, known as *Caryospora*-like, have been reported in *Isospora* infecting other mammals such as *I. canis, I. suis* and *I. rivolta.*[18-20] The presence of *Caryospora*-like oocysts of *I. belli* was first described in 1968 by Zaman who studied sporogonic development of *I. belli* from patients in Singapore.[2] However, no additional studies have confirmed the presence of this stage. In this study we demonstrated the presence of *Caryospora*-like oocysts in all 3 isolates examined after an extended period of incubation. Taken together, this stage could be an alternative ex vivo development of *I. belli*. Factors such as temperature, moisture, the level of oxygen and other unknown conditions could influence the

capability and duration required for complete sporulation of this type of oocysts.[1] For instance, the formation of *Caryospora*-like oocysts of cat isosporan protozoa, *I. rivolta*, could be induced by a brief heating of oocysts to 50°C before incubation. These oocysts remained viable and infectious to cats.[21] Although we did not determine the effect of heat induction for the generation of *Caryospora*-like oocysts in *I. belli*, it seems that spontaneous development of this stage occurred during a range of 5 days to 2 weeks at ambient temperature (25-30°C). If viability period of *I. belli* in environment after complete sporulation was limited, unsynchronized sporulation could extend the period of transmission of this important enteric coccidian protozoa.

Eukaryotic organisms possess the nuclear rRNA genes organized in clusters containing the SSU rRNA, 5.8S and 28S subunits that are separated by the two ribosomal RNA spacers, ITS1 and ITS2. A remarkable feature of these ITS sequences is their high divergence between species, rendering it a suitable marker for speciation and phylogenetic studies. Our analysis of the SSU rRNA, ITS-1, 5.8S rRNA and ITS-2 regions has revealed sequence conservation among isolates except a minimal sequence variation occurring in the SSU rDNA and ITS-1. Therefore, the existence of cryptic species of *Isospora* infecting patients in this study was unlikely.

Phylogenetic relationship inferred from the SSU rRNA gene has revealed that *Isospora* is more closely related to the genus *Neospora* and *Toxoplasma* than to the genus *Eimeria* and *Cyclospora* which is in agreement with previous analysis by Franzen and colleagues.[15] Likewise, the phylogenetic tree derived from the informative sites of ITS-2 based on secondary structure alignment yielded similar result to that obtained from the SSU rRNA sequence, supporting the value of ITS-2 data for tracing evolutionary relationship of coccidian protozoa as those used in analysis for other organisms.[22-24]

In conclusion, unlike *Cryptosporidium* infecting humans that comprises both zoonotic and anthroponotic species, our study demonstrated that human isosporiasis is caused by a single species belonging to *I. belli* based on morphometric and molecular evidences. Although isosporiasis is more common among immunocompromised patients than immunocompetent hosts, it seems likely that the severity of infections does not simply depend on the immune status of infected individuals because some immunocompetent patients exhibit chronic debilitating illness with multiple recurrent prolonged diarrheal episodes.[1,8] Whether strain difference in *I. belli* could contribute to disease severity will require further investigation. In this regard, analysis of yet unknown polymorphic genetic markers in *I. belli* would be essential.

References:

1. Lindsay DS, Dubey JP, Blagburn BL, 1997. Biology of *Isospora* spp. from humans, nonhuman primates, and domestic animals. *Clin Microbiol Rev 10:* 19-34.
2. Zaman V, 1968. Observations on human *Isospora. Trans R Soc Trop Med Hyg 62:* 556-557.
3. Morakote N, Muangyimpong Y, Somboon P, Khamboonruang C, 1987. Acute human isosporiasis in Thailand: a case report. *Southeast Asian J Trop Med Pub Hlth 18:* 107-111.
4. DeHovitz JA, Pape JW, Boncy M, Johnson WD, 1986. Clinical manifestations and therapy of *Isospora belli* infection in patients with the acquired immunodeficiency syndrome. *N Engl J Med 315:* 87-90.
5. Pape JW, Verdier RI, Johnson WD Jr, 1989. Treatment and prophylaxis of *Isospora belli* infection in patients with the acquired immunodeficiency syndrome. *N Engl J Med 320:* 1044-7.
6. Certad G, Arenas-Pinto A, Pocaterra L, Ferrara G, Castro J, Bello A, Nunez L, 2003. Isosporiasis in Venezuelan adults infected with human immunodeficiency virus: clinical characterization. *Am J Trop Med Hyg 69:* 217-222.
7. Brandborg LL, Goldberg SB, Breidenbach WC, 1970. Human coccidiosis: a possible cause of malabsorption. *N Engl J Med 283:* 1306-1313.
8. Jongwutiwes S, Sampatanukul P, Putaporntip C, 2002. Recurrent isosporiasis over a decade in an immunocompetent host successfully treated with pyrimethamine. *Scand J Infect Dis 34:* 859-862.
9. Tiangtip R, Jongwutiwes S, 2002. Molecular analysis of *Cryptosporidium* species isolated from HIV-infected patients in Thailand. *Trop Med Int Health 7:*357-364.
10. Morgan U, Weber R, Xiao L, Sulaiman I, Thompson RC, Ndiritu W, Lal A, Moore A, Deplazes P, 2000. Molecular characterization of *Cryptosporidium* isolates obtained from human immunodeficiency virus-infected individuals living in Switzerland, Kenya, and the United States. *J Clin Microbiol 38:*1180-1183.
11. Xiao L, Ryan UM, 2004. Cryptosporidiosis: an update in molecular epidemiology. *Curr Opin Infect Dis 17:*483-490.
12. Jongwutiwes S, Tiangtip R, Yentakarm S, Chantachum N, 2002. Simple method for long-term copro-preservation of *Cryptosporidium* oocysts for morphometric and molecular

analysis. *Trop Med Int Health 7:* 257-264.

13. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG, 1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res 25:* 4876-4882.
14. Siebert S, Backofen R, 2005. MARNA: multiple alignment and consensus structure prediction of RNAs based on sequence structure comparisons. *Bioinformatics 21:* 3352-3359.
15. Kumar S, Tamura K, Nei M, 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform 5:* 150-163.
16. Franzen C, Muller A, Bialek R, Diehl V, Salzberger B, Fatkenheuer G, 2000. Taxonomic position of the intestinal protozoan parasite *Isospora belli* as based on ribosomal RNA sequences. *Parasitol Res 86:* 669-676.
17. Bialek R, Binder N, Dietz K, Knobloch J, Zelck UE, 2002. Comparison of autofluorescence and iodine staining for detection of *Isospora belli* in feces. *Am J Trop Med Hyg 67:* 304-305.
18. Mahrt JL, 1968. Sporogony of *Isospora rivolta* oocysts from the dog. *J Protozool 15:* 308-312.
19. Lepp D, Todd KS, 1976. Sporogony of the oocysts of *Isospora canis*. *Trans Am Microsc Soc 95:* 89-103.
20. Linsay DS, Current WL, Ernst JV, 1982. Sporogony of *Isospora suis* Biester, 1934 of swine. *J Parasitol 68:* 861-865.
21. Matsui T, Ito S, Fujino T, Morii T, 1993. Infectivity and sporogony of *Caryospora*-type oocyst of *Isospora rivolta* obtained by heating. *Parasitol Res 79:* 599-602.
22. Ferrer C, Colom F, Frasés S, Mulet E, Abad JL, Alió JL, 2001. Detection and identification of fungal pathogens by PCR and by ITS2 and 5.8S ribosomal DNA typing in ocular infections. J Clin Microbiol 39: 2873–2879.
23. Young I, Coleman AW, 2004. The advantages of the ITS2 region of the nuclear rDNA cistron for analysis of phylogenetic relationships of insects: a Drosophila example. *Mol Phylogenet Evol 30:* 236-242.
24. Prakash A, Walton C, Bhattacharyya DR, Loughlin SO, Mohapatra PK, Mahanta J, 2006. Molecular characterization and species identification of the *Anopheles dirus* and *An. minimus* complexes in north-east India using r-DNA ITS-2. *Acta Trop 100:* 156-161.

Figures and Tables

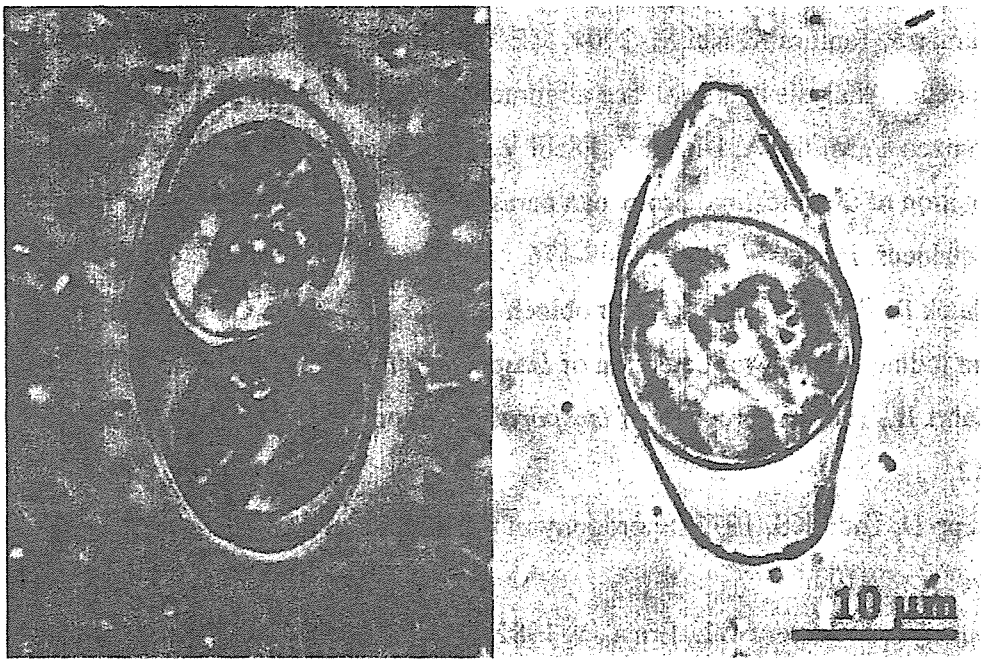

Figure 1 Oocysts of *Isospora belli* depicting sporulated oocyst containing two sporocysts, each of which contains 4 sporozoites (left) and *Caryospora*-like oocyst containing sporozoites enclosed in a single sporocyst (right).

```
I.belli          AAACTTTCAG CAATGGATGT CTTGGTTCGC GCAACGATGA AGGACGCAGC GAAATGCGAA ACGCAATGTG AATTGCAGAA  80
E.papillata      .......... .......... .....C.... ...T...... .......... .........T .....T.... .........T  80
Cy.cayetanensis  .......... .......... .....C.... ...T...... .......... .........T .....T.... .........T  80
T.gondii         ...T...... .......... .....C.... .......... .......... ...C...... .......... ..........  80
H.heydorni       ...T...... .......... .....C.... .......... .......... ...C...... .......... ..........  80
N.caninum        ...T...... .......... .....C.... .......... .......... ...C...... .......... ..........  80
C.parvum         .C.....A.. T......... ........T. AT........ .......... A..G.....T .A........ ..C....T..  80

I.belli          TTCAGTGAAT CATCAGATTT CTGAACGCAA ATGGCGCTGT GGGG-ATATT CCCTGCAGCA TGTCTGTTTC A-GTGTCTCT 158
E.papillata      .........C .......... .........T .........C T..--GAT.. .......... C......... .-......T. 157
Cy.cayetanensis  .........C .........C. .........T ..T......C C..CC..... G..G...... C......... .A......T. 160
T.gondii         .......... .......... .......... .....A.CT. ....-..... .T.CTTG.T. C......... .-......T. 158
H.heydorni       .......... .......... .......... .....A.CT. ....-..... .T.CTTG.T. C......... .-......T. 158
N.caninum        .......... .......... .......... ...C.A.CT. ....-..... .T.CTTG.T. C......... .-......T. 158
C.parvum         ...TA....C A.......C. ..C....... ..A..AG.A- --------... TA.ATT.CT. .A.T.A.A.. .-......A. 151
```

Figure 2 Alignment of the 5.8S rRNA sequences comparing *Isopora belli* with other coccidian protozoa. Dots and dashes identical represent residues and deletions, respectively.

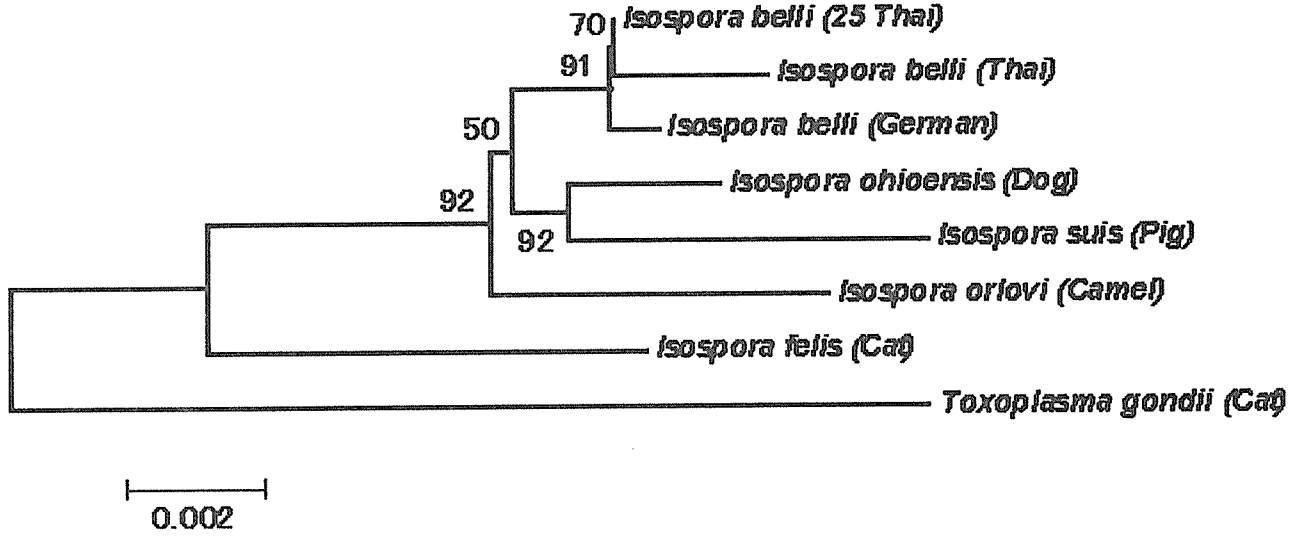

Figure 3 Phylogenetic relationships among isolates of *Isaspora belli* in this study (DQ060658-83) and those reported by others (U94787 and AF106935) in relation to other nonhuman species of *Isospora* as inferred from the SSU rDNA sequences and the neighbor-joining method. *Toxoplasma gondii* was used as the outgroup. Bootstrap percentages more than 50% based on 1,000 iterations are shown on the branches. Host origins are shown in parentheses.

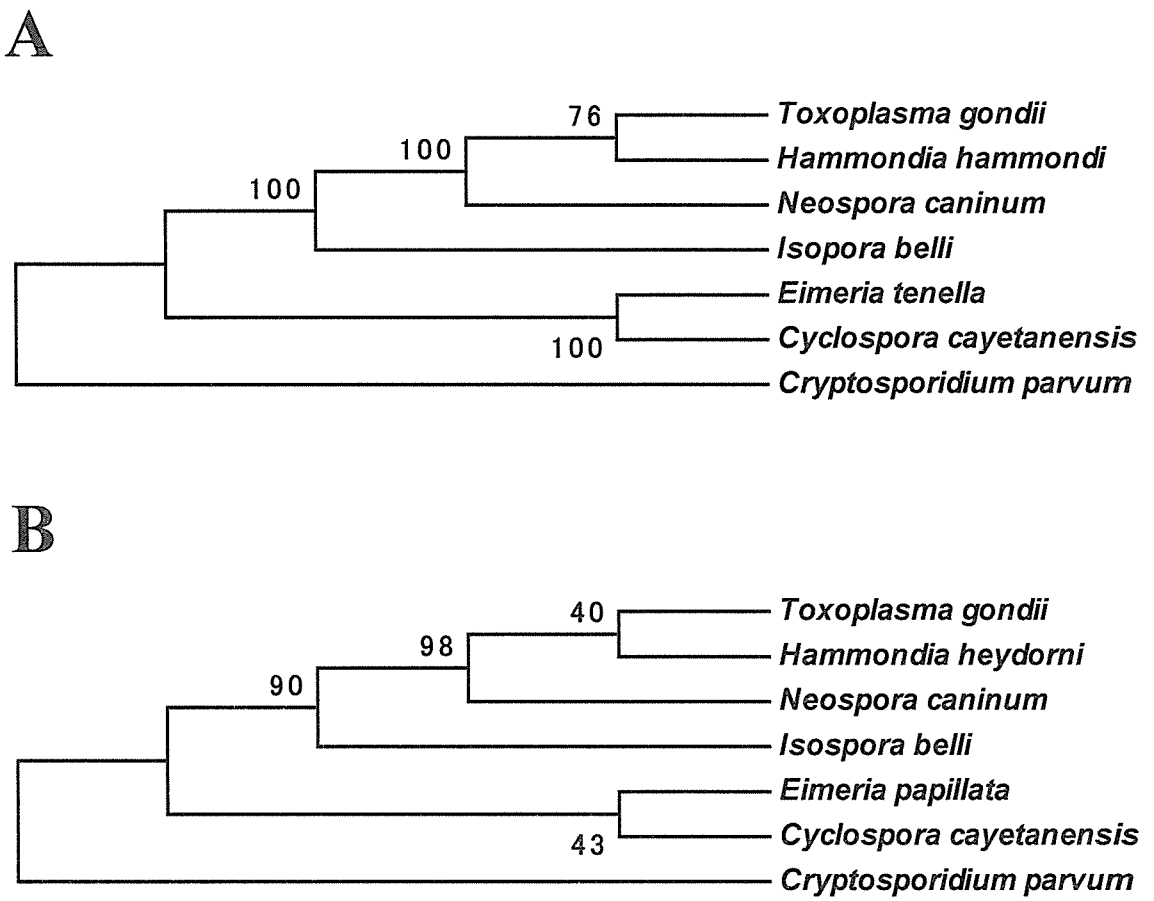

Figure 4 Topology of neighbor-joining trees derived from the SSU rRNA (A) and the ITS-2 region (B) of *Isospora belli* and other coccidian protozoa using Kimura-2-parameter. Bootstrap values based on 1,000 iterations are indicated on the branches.

Table 1 Clinical profiles of isosporiasis and morphometry of *Isospora belli* oocysts

Patient profiles	n	age (year) mean±SD (range)	CD4+ cell/μl mean±SD (range)	Eosinophil(%) mean±SD (range)	Oocyst dimension*		
					Length(μm) mean±SD (range)	Width(μm) mean±SD (range)	Shape Index mean±SD (range)
HIV infection (18 males, 12 females)							
Diarrhea							
≤3 weeks	5	35.6±11.0 (23-52)	104.3±25.7 (89-134)	5.4±3.6 (0.4-9.1)	27.2±2.6 (18-33)	12.8±1.8 (8-19)	2.2±0.3 (1.3-3.0)
>3 weeks - <1 year	22	36.7±6.9 (25-50)	60.4±52.9 (8-480)	4.5±3.3 (0.1-14.0)	28.4±2.9 (17-35)	13.7±1.9 (8-21)	2.1±0.3 (1.3-3.3)
≥1 year	3	27.7±8.3 (21-37)	80.3±53.6 (25-484)	3.5±1.8 (2.0-5.5)	28.4±3.8 (18-34)	13.8±2.2 (10-19)	2.1±0.4 (1.4-3.0)
Corticosteroid treatment (1 male, 2 females)							
No symptom	1	37	ND	0.8	33.7±2.0 (30-37)	14.0±1.6 (12-18)	2.4±0.2 (1.9-2.7)
Diarrhea							
≤3 weeks	1	51	ND	0	28.3±2.6 (23-32)	12.8±1.2 (10-15)	2.2±0.3 (1.8-3.1)
>3 weeks	1	23	ND	1.0	27.4±2.1 (23-30)	13.4±1.3 (11-16)	2.1±0.3 (1.4-2.6)
Immunocompetence (3 males, 2 females)							
No symptom	1	37	ND	12.0	28.0±1.9 (25-31)	14.0±1.9 (11-17)	2.0±0.3 (1.5-2.5)
Dyspepsia	1	31	ND	11.1	30.3±2.6 (25-35)	13.7±1.2 (12-16)	2.2±0.3 (1.6-2.5)
Diarrhea							
<1 year	2	30.5±2.1 (29-32)	ND	8.5±0.7 (8.0-9.0)	27.0±2.4 (20-32)	12.6±2.2 (9-18)	2.2±0.4 (1.5-3.1)
≥1 year	1	57	730	16.0	28.4±1.4 (26-30)	15.6±1.7 (12-17)	1.8±0.2 (1.6-2.3)

* Measurement under 400x magnification from 20 oocysts from each isolate. ND = not determined.

Title: MOLECULAR EPIDEMIOLOGY OF *BLASTOCYSTOSIS* AMONG INFECTED INDIVIDUALS IN THAILAND

Summary:

Although the pathogenic potential of *Blastocystis* remains inconclusive, the high prevalence of this protist detected in a number of stool surveys has attract medical attention. A large retrospective survey of this organism in stools of patients (n=108,851) seen at King Chulalongkorn Memorial Hospital in Bangkok from 2001-2005 has revealed that *B. hominis* was the most prevalent protozoan infection albeit most of the infected individuals did not have gastrointestinal illness. In order to analyze the genetic diversity in human isolates of *Blastocystis* among infected individuals in Thailand, we have recruited 70 stool samples harboring *B. hominis* for in vitro cultivation using a biphasic Boeck and Drbohlav's medium containing Locke's solution and egg slant. All isolates were successfully propagated in culture and the predominant stages were vacuolar forms, followed by granular forms while other stages such as multivacuolar and amoeboid forms were infrequently encountered. Sequence analysis of the small subunit ribosomal RNA gene of these isolates yielded 4 subtypes, i.e. subtypes I, II, III and V comprising 35.7, 2.8, 58.6 and 2.8%, respectively. There was no significant association between particular subtypes and gastrointestinal symptoms of infected individuals. Further analysis using more isolates will be performed to elucidate the extent of genetic diversity among isolates of *B. hominis* among Thai population.

Introduction

Blastocystis hominis is a single-celled eukaryotic organism that has been positioned within the stramenopile protists, being most related to *Proteromonas lacertae*, a flagellate parasite of reptiles [1]. Frequently encountered developmental stages of *B. hominis* comprise vacuolar, granular, amoeboid and cyst forms. Other stages that are less commonly found include avacuolar and multivacuolar forms, and cells containing filamentous-like inclusions. The cystic stage of *B. hominis* is thought to play a significant contribution of its mode of transmission [2.3]. Viability studies revealed that the cysts can survive at room temperature for more than 2 weeks and withstand lysis by water. In contrast, the vacuolar and granular forms become fragile during temperature changes, hypotonic and hypertonic environments and exposure to air [1,2].

Human infections with *B. hominis* occur worldwide and the prevalence approached more than half of the population in some surveys. Prevalence in humans in developed countries is

variable ranging from 0.5% to 23% as detected in Japan and the United States, respectively. Although most of the *B. hominis*-infected individuals remain asymptomatic, in certain circumstances infection with this organism has been linked to diarrheal illness and irritable bowel syndrome [4,5]. Meanwhile, *B. hominis* isolated from humans and animals exhibits morphological similarity while genetic and antigenic profiles of these organisms display remarkable variation. Furthermore, molecular analysis of the small subunit ribosomal RNA has revealed that sequence variation occurs among human isolates and that some isolates share sequence identity with those isolated from animals, indicating zoonotic transmission potential and lack of host-specificity of this protest [6-8]. Despite significant difference in the small subunit ribosomal RNA sequences and diverse karyotypic profiles that could potentially classify *Blastocystis* into distinct species, to date conclusive nomenclature of the species has not been universally described [6].

The pathogenic properties of *Blastocystis* remain unknown due to the lack of good animal models and limited knowledge of the extent of diversity among a large population of parasites, rendering the comparative analysis of genotypic differences between infected individuals with diarrheal illness and those without diarrhea. In addition, the presence of specific strains/genotypes of the organisms that are shared between humans and animals would indirectly indicate the zoonotic potential of this protest [2]. In this study, we analyze the small subunit ribosomal RNA gene of *Blastocystis* isolated from patients who attended King Chulalongkorn Memorial Hospital in Bangkok, Thailand.

Purposes:

1. To determine the prevalence of *Blastocystis* among Thai patients at King Chulalongkorn Memorial Hospital in Bangkok, Thailand.
2. To analyze intraspecific variation in human isolates of *Blastocystis* based on the small subunit ribosomal RNA sequences.

Methods:

Study population

To determine the prevalence of *B. hominis*, we did a retrospective survey of parasitic infections of patients at The King Chulalongkorn Memorial Hospital in Bangkok who submitted their stool samples for parasitological diagnosis by direct smear and formalin-ethylacetate sedimentation methods. The survey period was retrospectively performed from January 2001 through December 2005.

Blastocystis isolates and *in vitro* propagation

Stool samples from infected individuals containing *Blastocystis* as determined by either direct smear or formalin-ethylacetate sedimentation method were subject to *in vitro* propagation using Boeck and Drbohlav's medium containing Locke's solution (sodium chloride 8.0 g/l, calcium chloride 0.2 g/l, potassium chloride 0.2 g/l, magnesium chloride 0.2 g/l, dibasic sodium phosphate 2.0 g/l, sodium bicarbonate 0.4 g/l and monobasic potassium phosphate) and sterile egg slant. After applying approximately 0.1-0.2 g of fecal to 15 ml of medium in 20 ml glass tube with cap, the culture was incubated at 37°C and aliquot of liquid part was prepared on glass slides to search for *Blastocystis* under light microscope at 24 hour interval for 5 days. This study has been approved by the Ethical Review Committee of Research, Faculty of Medicine, Chulalongkorn University.

DNA extraction

Isolation and purification of DNA from *Blastocystis* were done by the method by using QIAamp DNA stool mini kits (QIAGEN, Germany). The procedures were essentially performed following the manufacter's instruction.

Amplifications of the small subunit ribosomal RNA gene

Amplification of the small subunit ribosomal RNA gene of *Blastocystis* was performed by polymerase chain reaction (PCR) in 20 microliters reaction mixture containing 200 microM each dNTP, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 1.25 units of ExTaq DNA polymerase (Takara, Japan), *Blastocystis* DNA and 0.2 mM of each primer. The forward primer was Bh-F0: 5'- GCTTATCTGGTTGATCCTGCCAG-3'and the reverse primer Bh-R0: 5'-TGATCCTTCCGCAGGTTCACC-3'which span 1.7 kilobases of the small subunit ribosomal RNA gene. The thermal cycler profile contained 35 cycles of 94, 60 and 72°C for 30, 40 and 120 s, respectively. The PCR amplified products were analyzed by electrophoresis on 1% agarose gel, stained with ethidium bromide and visualized under UV transilluminator.

DNA sequencing

DNA sequence was determined from PCR-amplified products which were purified by QIAquick PCR Purification kit (QIAGEN). DNA sequencing was performed directly from two independent PCR products without cloning, using the Big-Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing primers were designed to obtain overlapping target regions in both directions. A singleton polymorphism was verified by sequencing the PCR products from a new amplification reaction using the same DNA template.

Statistical analysis

Alignment of nucleotide sequences was done by using the clustal X program and by

visual inspection.

Results

Prevalence of *Blastocystis* in Thailand

From 2001 to 2005, the Laboratory of Parasitology of King Chulalongkorn Memorial Hospital in Bangkok, the second largest hospital in Thailand accepted 108,851 stool samples for a routine parasitological screening by both direct smear and concentration method. Of these, 10.28% (11,194 samples) were positive for one or more parasites. The most common protozoan infection was *B. hominis* comprising 25.17% of all positives, followed by *Giardia intestinalis* (11.31%) while *Strongyloides stercoralis* was the most prevalent helminthic infections (23.58%), followed by *Opisthorchis viverrini* and hookworm, respectively (Table 1). There was no significant difference in sex of patients who were infected with *B. hominis* (data not shown).

In vitro cultivation of *Blastocystis*

In the initial step, we collected 70 fecal samples that were positive for *B. hominis* for *in vitro* propagation prior to DNA extraction. The majority of isolates were from female patients and most of the isolates were from patients without diarrhea. All samples could be successfully cultivated, resulting in the presence of several developmental stages in culture (Figure 1). The most commonly encountered stage was vacuolar form, characterized by a spherical cell with a large central body and a thin peripheral band of cytoplasm surrounding the central body. The granular form resembles the vacuolar form but the central body contains granules of several morphological types. Other forms that occur relatively rare include amoeboid and multivacuolar forms. However, cystic stage has not been identified in cultures of all isolates in this study.

Intraspecific variation of *Blastocystis* based on the small subunit ribosomal RNA gene

Among 7 known subtypes of *B. hominis*, we detect 4 subtypes in Thai patients. The sequences of each subtype were shown in Figure 2. Subtype III was most prevalent, representing more than half of all isolates examined. Twenty-five isolates (35.7%) contained *Blastocystis* subtype I while subtypes II and VI were identified in 2 isolates each. We could not detect subtypes IV, VI and VII in any isolates examined (Table 2). There is no tendency toward the correlation between particular subtypes and diarrheal symptom of the patients.

Discussions

Several epidemiological studies have shown that *B. hominis* are prevalent in people from both developing and developed countries [2,5]. Our retrospective analysis of a large number of

samples from patients attending King Chulalongkorn Memorial Hospital in Bangkok has re-affirmed that *B. hominis* was the most prevalent protozoan parasite in Thai patients. In this study, we recruited a total of 70 stool samples from patients with diarrhea (13 cases) and those without diarrheal symptom (56 cases) who harbored *B. hominis* for genetic analysis. All of these samples could be propagate in culture, rendering further genetic analysis more feasible.

Blastocystis can infect a wide range of animals and humans. Analysis of the small subunit ribosomal RNA has shown considerable sequence variations among isolates. However, phylogenetic analysis has identified at least 9 monophyletic clades among *Blastocystis* from various hosts, ranging from insects to mammals [7]. It is interesting to note that there was no specific host preference for each clade but rather that *Blastocystis* in each clade may infect more than one host types. It is evident that there is extensive genetic diversity among *Blastocystis* isolates and therefore zoonotic potential of any isolate from non-human origins [2,9].

So far human isolates of *Blastocystis* can be classified into 7 subtypes. Our direct sequencing analysis of the small subunit ribosomal RNA sequence from PCR amplified DNA has detected 4 subtypes, i.e. subtypes I, II, III and V. Nevertheless, it is not unusual to find mixed infection of these same organisms which harbor different genotypes. Therefore, our next purpose is to reanalyze all of the isolates in this study by subcloning the PCR-amplified DNA into plasmid vector, followed by sequencing. This strategy will facilitate the detection of clonal mixture in a single isolate that cannot be entirely excluded from our present analysis. Nevertheless, this study will incorporate more samples in future analysis in order to explore the extent of sequence variation of the small subunit ribosomal RNA or related genes [10]. The knowledge on molecular epidemiology of *Blastocystis* could pave the way for better understanding of this perplexing organism along with eluciadation of its potential roles in pathogenesis of diarrheal disease [11-14].

References

1. Tan KS, Singh M, Yap EH. Recent advances in *Blastocystis hominis* research: hot spots in terra

incognita. Int J Parasitol 2002;32:789-804.
2. Tan KS. *Blastocystis* in humans and animals: new insights using modern methodologies.Vet Parasitol 2004;126:121-44.
3. Tan TC, Suresh KG. Predominance of amoeboid forms of *Blastocystis hominis* in isolates from symptomatic patients. Parasitol Res 2006;98:189-93.
4. Boreham RE, Benson S, Stenzel DJ, Boreham PF. *Blastocystis hominis i*nfection. Lancet 1996;348:272-3.
5. Cirioni O, Giacometti A, Drenaggi D, Ancarani F, Scalise G. Prevalence and clinical relevance of *Blastocystis hominis* in diverse patient cohorts. Eur J Epidemiol 1999;15:389–93.
6. Yoshikawa H, Morimoto K, Wu Z, Singh M, Hashimoto T. Problems in speciation in the genus *Blastocystis*. Trends Parasitol 2004;20:251-5.
7. Stensvold CR, Suresh GK, Tan KS, Thompson RC, Traub RJ, Viscogliosi E, Yoshikawa H, Clark CG. Terminology for *Blastocystis* subtypes: a consensus. Trends Parasitol 2007;23:93-6.
8. Arisue N, Hashimoto T, Yoshikawa H. Sequence heterogeneity of the small subunit ribosomal RNA genes among *Blastocystis* isolates. Parasitology 2003;126:1–9.
9. Clark CG. Extensive genetic diversity in *Blastocystis hominis*. Mol Biochem Parasitol 1997;87: 79–83.
10. Ho LC, Armugam A, Jeyaseelan K, Yap EH, Singh M. *Blastocystis* elongation factor-1 alpha: genomic organization, taxonomy and phylogenetic relationships. Parasitology 2000;121:135–44.
11. Giacometti A, Cirioni O, Fiorentini A, Fortuna M, Scalise G. Irritable bowel syndrome in patients with *Blastocystis hominis* infection. Eur J Clin Microbiol Infect Dis 1999;18:436–9.
12. Guglielmetti P, Sansoni A, Fantoni A, Rossolini A. Pathogenesis of blastocytosis. Lancet 1991;338: 57.
13. Nagler J, Brown M, Soave R. *Blastocystis hominis* in inflammatory bowel disease. J Clin Gastroenterol 1993;16:109–12.

14. Nimri L, Batchoun R. Intestinal colonization of symptomatic and asymptomatic schoolchildren
with *Blastocystis hominis.* J Clin Microbiol 1994;32:2865–6.