prolonged period of symptoms in patients with acquired immunodeficiency syndrome (AIDS) than in immunocompetent individuals. Among immunocompetent patients with isosporiasis, infants and young children seem to have more severe manifestations than in adults. Meanwhile, chronic diarrheal symptoms persisting for more than a decade caused by *I. belli* infections have been reported in adults without concurrent immunosuppression. Although the gastrointestinal symptoms of patients with isosporiasis usua11y subside within a few days after initiation of treatment with combination of trimethoprim and slfamethxazole, relapses have been observed in approximately half of symptomatic cases. Thus, the spectrum of isosporiasis seems to be variable, partly depending on the host immune status whereas variation in virulence of parasite strains remains unknown.

A number of distinct specie shave been characterized for *Cryptosporidium*, another enteric coccidian protozoa that causes diarrheal illness in both humans and animals. Recent molecular epidemiologic studies have shown that cryptosporidia infecting humans belong to heterogenous species. Most of these species are responsible for zoonotic transmission, and anthroponotic cryptosporidiosis is caused by *C. hominis*. The knowledge of speciation in cryptosporidia is of considerable importance for disease control and prevention, especially for those with an immunosuppressive status because no documented effective anti-cryptosporidial drugs available.

Despite the increased significance of enteric coccidiosis, comparatively little is known about species/strain variation in the genus Isospora infecting humans. To address this issue, we extracted the DNA from oocysts found in fecal samples of isosporiasis patients in Thailand and determined the sequences of the genes for small subunit ribosomal RNA (SSU rRNA),internal transcribed spacer (ITS), and 5.8 S ribosomal RNA. We also analyzed morphologic features of oocysts of *I. belli* from patients with normal immune status and those with compromised immunity.

MATERIALS AND METHODS

Stool samples and background data: We obtained stool Samples positive for *I. belli* oocysts from individuals at King Chulalongkorn Memorial Hospital in Bangkok, Thailand, as a cross-sectional study between January 2002 and December 2004. Isosporiasis was diagnosed by the presence of characteristic oocysts in one or more stool samples by direct wet smear method, formalin-ethylacetate sedimentation, or modified Kinyoun acid fast stain. Stool samples and clinical data of *I. bell* -infected cases were obtained after informed consent was obtained. These data included age, sex, presenting symptoms, history of illness, and relevant laboratory test results such as complete blood count and CD4+ lymphocyte count.

Eosinophilia was determined based on a relative eosinophil count of more than 4 cells per 100 leukocytes. Diarrhea was

defined as three or more unformed stools per day.

Aliquots of each *I. belli*-positive stool sample were preserved as described by adding approximately four volumes of absolute ethanol and storing at ambient temperature. Whenever possible, fresh stool samples were collected for observing sporulation of oocysts. The ethical aspects of this study were reviewed and approved by the Institutional Review Board of Faculty of Medicine, Chulalongkorn University.

DNA extraction: DNA of *I. belli* oocysts was extracted from either fresh stool samples or ethanol-preserved specimens by the method described for the extraction of Cryptosporidium oocysts, except that the Q7Aamp DNA Stool Mini Kit((Qiagen, Hilden, Germany) was used. Prior to DNA extraction of preserved samples, ethanol was removed by washing the samples with sterile water (by centrifugation 3 times at 1,600×g for 15 min without stopping) and the pellet was resuspended in 5 mL of water. The DNA purification procedure was essentially as described in the manufacturer's instruction manual. The purified DNA was dissolved in TE buffer (10mM Tris-HCl pH8·0, and 1 mM EDTA) and stored at 20°C until used.

Polymerase chain reaction (PCR): The DNA fragment spanning the SSU rRNA,ITS-1, 5.8S rRNA and ITS-2 region of *I. belli* was amplified by a nested PCR using primers whose sequence were derived from the 5′ portion of the SSU rRNA gene of *I. belli* isolate Cl l (GenBank accession no. U94787) and the 5′ portion of the 28S rRNA gene of *I. belli* (GenBank accession no. U85705). Sequences of the outer pair of primers were Iso-18SF0, 5′-CTGGTTGATCCTGCCAGTA-3′and Iso-28SR0, 5′-AAGGCTCAATCAAr GAACCTCCG-3′. Sequences of the inner pair of primers were Iso-1SSF1, 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 for 40 seconds, annealing at 64°C for 40 seconds, extension at 74°C for 5 minutes for 35 cycle 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 (Takara, Shiga, Japan) that has efficient 5′ → 3′ exonuclease activity to increase fidelity and shows no strand displacement. The size of PCR product was examined by electrophoresis in a 1% agarose gel and visualized with an ultraviolet Transilluminator (Mupid Scope WD, Japan). The PCR product was purified by using the QIAquick PCR purification kit (Qiagen).

Subcloning: The GeneJETTM PCR Cloning Kit was used for ligating the purified PCR product to the plasmid vector pJETl/blunt (Fermentas, Burlington, Ontario, Canada). After incubation at 15°C for 6 hours, the

reaction mixture was precipitated, re-dissolved in 10μl of double-distilled water, and transformed into *Escherichia coli* strain JM107 by electroporation using an E. coli pulsar apparatus (Bio-Rad Laboratories, Hercules, CA). Recombinant DNA from positive clones was prepared by using the QIAGEN plasmid minikit (Qiagen).

DNA sequencing: DNA sequences were determined directly from both PCR-purified templates and plasmid sub-clones. Sequencing analysis was performed in both directions for each template using the Big DyeTerminator version 3.1 Cycle Sequencing Kit on anAB1310 Genetic Analyzer (Applied Biosystems). Overlapping sequences were obtained by using sequencing primers (available upon request). Whenever one substitution occurred, the 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 . Phylogenetic construction was performed by the neighbor-joining method using the Kimura-2 parameter and 1,000 bootstrap iterations as implemented in the MEGA version 3.1 program. To analyze the evolutionary relationship, homologous sequences of other coccidian parasites available in the GenBank database were incorporated for comparison: *I. belli* (AFlO6935), *I. ohioensis* (AF029303), *I. suis* (U9753), *I. orlovi* (AY365026), *Cyclospora cayetanensis* (U40261 and AF301391), *Neospora caninum* (U16159 and L49389), *Hammondia heydroni* (AF317282), *H. hammondi* (AF094698), *Eimeria tenella* (AF317282) and *E. papillata* (AY779509). New nucleotide sequence data obtained in this study have been deposited in the GenBank database under accession nos. DQ060658 - Q060683.

RESULTS

Patient profiles: Between January2002 and December 2004, stool samples from 39,510 individuals were submitted to parasitology laboratory of King Chulalongkorn Memorial Hospital in Bangkok for detection 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 136 by modified Kinyoun acid fast stain (94.7%). Isosporiasis patients comprised 35 Thai persons from diverse regions of the country and 3 recent immigrants from Cambodia, Laos, and Pakistan.

Of all *I. belli*-infected patients, 30 were HIV positive,3 received prolonged corticosteroid therapy for systemic lupus erythematosus idiopathic thrombocytopenic purpura, and 5 were immunocompetent individuals. More than half of isosporiasis patients were 30-39 years of age and the ratio of male to females

1.38:1. Laboratory tests showed relative eosinophilia (> 4%) in half of HIV-positive cases and all immunocompetent patients but none in the patients receiving prolonged corticosteroid treatment. The absolute CD4+1yrnphocyte counts in HIV-positive cases were 8 - 484 cells/μl (mean ± SD = 67.5 ± 57.4 ce11s/μl).

Table Clinical profiles of isosporiasis

Patient profiles	n	Age (years) mean ± SD (range)	CD4+ cells/μL mean ± SD (range)	Eosinophils (%) 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)
> 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)
≥ 1 year	3	27.7 ± 8.3 (21–37)	80.3 ± 53.6 (25–484)	3.5 ± 1.8 (2.0–5.5)
Corticosteroid treatment (1 male, 2 females)				
No symptom	1	37	ND	0.8
Diarrhea				
≤ 3 weeks	1	51	ND	0
> 3 weeks	1	23	ND	1.0
Immunocompetence (3 males, 2 females)				
No symptoms	1	37	ND	12.0
Dyspepsia	1	31	ND	11.1
Diarrhea				
< 1 year	2	30.5 ± 2.1 (29–32)	ND	8.5 ± 0.7 (8.0–9.0)
≥ 1 year	1	57	730	16.0

* HIV = human immunodeficiency virus; ND = not determined.

Among HIV infected cases, concurrent infections with one or more of the following pathogens occurred in 9 patients: herpes simplex virus, herpes zoster virus, *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 ± SD = 3.3 ± 3.4 months) prior to attending King Chula1longkorn Memorial Hospital. Two patients who received corticosteroids had chronic watery diarrhea and one patient who received this treatment had no symptoms. The clinical profiles of isosporiasis in immunocompetent patients were asymptomatic in two patients, chronic watery diarrhea in two patients, and

dyspepsia without diarrhea in one patient. Diarrhea and associated gastrointestinal symptoms resolved within a few days after initiation of treatment with a combination of trimethoprim and sulfamethoxazole. During two months of follow-up, relapse occurred once each in two HIV-infected patients and one immunocompetent patient that was controlled by long term prophylaxis with trimethoprim and sulfamethoxazole.

SSU rRNA, ITS-1, 5.8S rRNA and ITS-2 sequences: The DNA fragment generated from the secondary PCR encompassing the SSUrRNA,ITS-1, 5.8S rRNA,and ITS-2 genes contained 3,049 base pairs. All isolates yielded single PCR 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%). Sequences of the SSUrRNA gene, spanning, 778 base pairs 25 isolates examined were identical with those of strains CI1 and CJLPHD2 (GenBank accession nos. U94787 and AF441289), but differed from the isolate reported by Franzen and others (GenBank accession nos. AFlO6935) at A679T and A682C. Three additional nucleotide substitutions occurred at T583C,C638A,and G1240T in the isolate from immunocompetent patient who had multiple relapses. The 5.8 S 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* showed perfect sequence identity among all isolates examined. Likewise, sequences of the ITS-1 were highly conserved, except for one nucleotide substitution at position 528 with an A to G change (position according to the ITS-1 region). However, there was no association between this nucleotide substitution and clinical severity of the infected individuals. Comparison of the 5.8S RNA of I. belli with those of other coccidian parasites showed a high similarity of sequences. Conversely, the ITS-1 and ITS-2 regions showed 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, which is consistent with a single species. Further analysis using the available complete sequences of the SSU rRNA gene of the genera *Toxoplasma, Cryptosporidium, Cyclospora, Isospora, Neospora, Eimeria,* and *Hammondia,* which were aligned according to their secondary structure, showed that *I. belli* was more related to *Toxoplasma, Neospora,* and *Hammondia* than to *Cyclospora, Eimeria,* and *Cryptosporidium.* An identical topology of a phylogenetic tree to that inferred from the SSU rRNA sequences was reaffirmed when the ITS-2 sequences were used for comparison.

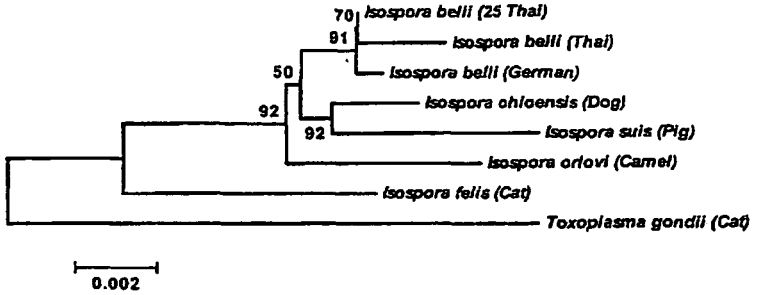

FIGURE Phylogenetic relationships among isolates of *Isospora belli* in this study (DQ060658-83) and those reported by others (U94787 and AF106935) in relation to other nonhuman species of *Isospora* as inferred from small subunit ribosomal RNA sequences and the neighbor-joining method. *Toxoplasma gondii* 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.

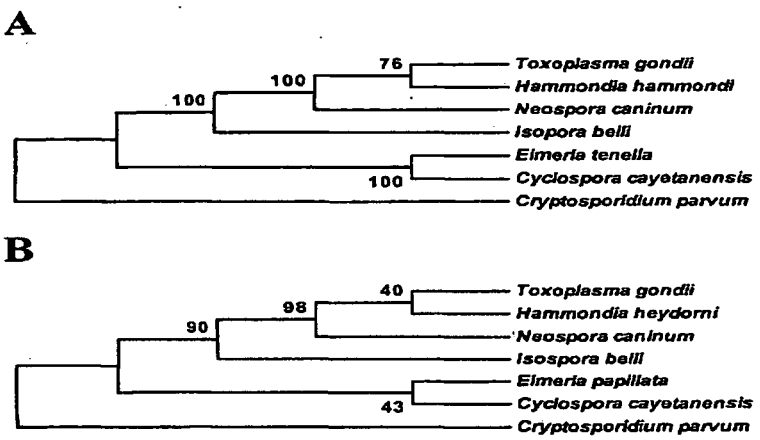

FIGURE Topology of neighbor-joining trees derived from the small subunit ribosomal RNA (**A**) and the internal transcribed spacer 2 region (**B**) of *Isospora belli* and other coccidian protozoa using the Kimura-2 parameter. Bootstrap values based on 1,000 iterations are indicated on the branches.

DISCUSSION

We searched for isosporiasis in a large number of patients who submitted their stool samples to King Chulalongkorn Memorial Hospital over a three-year period. The overall prevalence of *I. belli*-positive samples was approximately 0.1% on the basis of 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%. The low prevalence of isosporiasis in this study suggested that I. belli might not commonly circulate in the

Thai population, resulting in a low prevalence of isosporiasis in HIV/AIDS patients in this country. Nevertheless, most isosporiasis patients in Thailand showed immunosuppression, comprising 86.8% of all positive cases. Despite rapid improvement of symptoms after treatment with oral trimethoprim and sulfamethoxazole, recurrent isosporiasis occurred in three HIV/AID patients (approximately 8%) within two months after initiation of treatment. Importantly, about half of Haitian patients who received the same or similar treatment had relapses. The duration and frequency of follow-up after treatment may have contributed to the difference in observed relapsing episodes from each study. However, continuing anti-coccidial prophylaxis will be warranted in certain isosporiasis cases.

Eukaryotic organisms possess the nuclear rRNA genes organized in clusters containing SSU rRNA, of which the 5.8S and28S subunits are separated by the two ribosomal RNA spacers ITS1 and ITS2. A remarkable feature of these ITS sequences is their high divergence between species. which makes them a suitable marker for speciation and phylogenetic studies. Our analysis of the SSU rRNA,ITS-1,5.8S rRNA, and ITS-2 regions showed sequence conservation among isolates, except a minimal sequence variation in SSU rDNA and ITS-1. Therefore, cryptic species of Isospora infecting patients in this study were unlikely.

Phylogenetic relationships inferred from the SSU rRNA gene showed that Isospora is more closely related to the genera Neospora and Toxoplasma than to the genera Eimeria and Cyclospora, which is consistent with analysis by Franzen and others. Likewise, the phylogenetic tree derived from the informative sites of ITS-2 based on secondary structure alignment yielded results similar to those obtained from the SSU rRNA sequence, which supports the value of ITS-2data for tracing evolutionary relationship of coccidian protozoa as those used in analysis of other organisms.

In conclusion, unlike *Cryptosporidium* infecting humans that comprises both zoonotic and anthroponotic species, our study, on the basis of Morphometric and molecular evidence, showed that human isosporiasis is caused by one species of *I. belli*. Although isosporiasis is more common among immunocompromised patients than immunocompetent hosts, it seems likely that severity of infections does not simply depend on the immune status of infected individuals because some immunocompetent patients exhibit chronic debilitating i11ness with multiple recurrent prolonged diarrheal episodes. 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.

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Prevalence of *Giardia* and *Cryptosporidium* in Stool Samples of Diarrheic Patients from the Philippines

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ABSTRACT

The prevalence of *Giardia* and *Cryptosporidium* in 3456 diarrheic patients in the Philippines was determined. Out of 133 (3.85%) positive samples, 69 (2.00%) were positive for *Giardia* and 67 (1.94%) for *Cryptosporidium*. Three samples had co-infection of *Giardia* and *Cryptosporidium*. Luzon had the highest positive samples (4.98%) followed by Mindanao (4.87%), then Visayas (2.22%). *Giardia* was most prevalent in Mindanao (3.59%) while *Cryptosporidium* was most prevalent in Luzon (3.12%). The prevalence of *Giardia* (1.99%) among pediatric patients (0-18 yrs) did not significantly differ from that (1.93%) among adults (>18 years old). However, for *Cryptosporidium*, the prevalence (2.92%) among pediatric patients was significantly higher compared to that (0.24%) among adults. The prevalence of *Giardia* but not of *Cryptosporidium* was significantly higher in male than in female adults. For both protozoa, prevalence was significantly higher in pediatric than in adult females. Significantly higher prevalence of *Giardia* and *Cryptosporidium* was found in 4 to 9 and 0 to 3 years old, respectively. Seasonality had a distinct peak in September with *Cryptosporidium* more prevalent in the rainy (2.59%) than during the dry season (0.89%).
Using PCR-RFLP and sequencing, 3 species of *Cryptosporidium* (*C. hominis*, *C. parvum*, and *C. canis*) were identified from 22 samples that were positive by microscopy.

INTRODUCTION

Diarrhea is considered a major cause of morbidity, especially in developing countries. In the Philippines, it was the leading cause of morbidity for the years 2001 and 2003, and the second in 2002 (1). Common causes of diarrhea are infections due to viruses, bacteria, helminthes and protozoa. These causative agents are either food-borne or water-borne.

Among enteric protozoa, *Giardia lamblia* (syn. *G. intestinalis* or *duodenalis*) and *Cryptosporidium* spp. are the most commonly reported causes of waterborne diarrhea outbreaks.

G. lamblia is especially prevalent in children in developing countries (2), and is the most commonly diagnosed flagellate in international travel (3). So far, all outbreaks for *Cryptosporidiumsis* have been associated with waterborne transmission.

G. lamblia was reported in the Philippines by Cross and his co-workers in 1977 (4) and since then, it has been identified as a common intestinal parasite. Studies done in Luzon (5-9), in various localities in the Visayas (4, 10, 11), and in the southern islands of Mindanao (12) show that *Cryptosporidium* is widely distributed in the Philippines. It has also been reported among children living in various residential institutions (13, 14), and among measles patients with diarrhea (15).

Cryptosporidium is a coccidian protozoan pathogen that can cause life-threatening diarrhea in an immunocompromised host. Following the first report on cryptosporidiosis in Philippine children made by Cross et al. in 1985 (16), local studies on *Cryptosporidium* as an etiologic agent for diarrhea have focused on its prevalence in children (8, 15, 17-21). A recent study by Rivera et al. (22) detected *Cryptosporidium* antibodies among Filipino cancer patients.

Stool examinations in the Philippines typically include the identification of the common etiologic agents of diarrheas such as rotavirus and bacteria (*E. coli, Shigella, Campylobacter, Salmonella,* and *Vibrio cholerae*). In major tertiary hospitals in the Philippines, routine stool examinations may include *G. lamblia,* which can be readily identified by microscopy. However, identification of *Cryptosporidium* is not routinely done, unless specifically indicated by a physician.

Molecular studies allow species identification of *Cryptosporidium.* There are at least 7 species which have been found pathogenic in humans (23). The three most commonly reported are *C. parvum, C. hominis,* and *C. meleagridis.* In this study, we determined the species of 22 isolates which were morphologically identified as *Cryptosporidium.*

This work is the most recent nationwide survey of *Giardia* and *Cryptosporidium* and provides basic information on the prevalence of these enteric protozoa in the Philippines. This is the first report of molecular characterization of *Cryptosporidium* in the country.

MATERIALS AND METHODS

Patients: Stool samples were collected from patients who consulted for diarrhea in collaborating hospitals and health centers from May 2004 to May 2005 in the three main islands of the Philippines. There were 31 collaborators from Luzon, 39 from Visayas, and 9 from Mindanao.

Demography: Patients or relatives were asked to fill up an information sheet that provided the demographic data for this work.

Ethical considerations: This project was given ethical clearance by the St. Luke's Institutional Ethics Review Board, and the required informed consent was obtained from patients or their relatives.

Stool collection and processing: Single fecal samples were collected from each patient and 1 ml of each sample was placed in a 15 ml polypropylene tube that contained 9 ml

of 10% formalin. The fixed fecal samples were stored at 4°C until these were transported to the Research and Biotechnology Division of St. Luke's Medical Center, Quezon City Philippines, for processing and microscopic examination.

All formalin-fixed stool specimens were concentrated using the formalin-ethyl acetate method. To detect *Giardia* and *Cryptosporidium*, 5 µl from each stool concentrate and 5 µl of detecting antibodies from the MeriFluor® *Cryptosporidium-Giardia* direct fluorescence detection kit (Meridian Diagnostics, Inc., Cincinnati, Ohio) were mixed on a slide.

Microscopy: Each slide was scanned under a 20x objective (Zeiss Axiolab microscope). *Giardia* and *Cryptosporidium* showed apple-green fluorescence with a blue excitation filter of 450 nm (09B, Zeiss). *Giardia* cysts were oval, measuring approximately 11-14 µm, while *Cryptosporidium* oocysts were round and smaller (4-6 µm). To further observe cyst/oocyst morphology, bright field observation was also done with a 100x objective.

Statistical Analysis: Data from the information sheets were encoded in Microsoft Excel. Data processing and analysis were performed using SPSS ver. 14 software. Descriptive statistics such as means and proportions were used to describe the patients' socio-demographic characteristics. The Chi-square or Fisher's Exact and t-test statistics were used to test for differences in distribution. All tests were two-tailed and considered significant at $p<0.05$.

Oocyst disruption and DNA extraction: Genomic DNA was extracted from *Cryptosporidium* isolates identified based on fluorescence microscopy. Oocysts were purified from the stool samples by formalin-ethyl acetate concentration method. Genomic DNA of *Cryptosporidium* was isolated from the purified oocysts by QIAamp DNA stool minikit (QIAGEN Ltd., West Sussex, United Kingdom) following the manufacturer's instructions.

Alternative DNA extraction method was performed as follows: approximately 200 µl of purified oocysts in sterile Tris-EDTA (TE) buffer was added to 100 mg of 0.5-mm glass beads plus 400 µl of 1% sodium dodecyl sulfate (SDS). The tube was vortexed for two minutes at maximum speed, boiled for 30 minutes, and incubated at 56°C overnight after adding 5 µl of proteinase K (10 mg/ml). DNA was isolated by adding 400 µl of buffered phenol and spinning at 12,000 rpm for five minutes at 27°C. DNA was precipitated by adding 40 µl of sterile 1 M sodium chloride (NaCl) and 800 µl of absolute ethanol. The tube was centrifuged at 14,000 rpm for five minutes at 27°C. The supernatant was discarded while the pellet was washed with 400 µl of 70% ethanol and centrifuged at 14,000 rpm for five minutes at 27°C. The pellet was dried at room temperature. The DNA was eluted into 25 µl sterile TE buffer. DNA sample was either used directly for PCR amplification or stored at -20°C until used.

Analysis of *Cryptosporidium* spp. from the Philippines: For the genotyping of *Cryptosporidium* spp., PCR was performed to amplify a region in the polythreonine gene (24). Nested PCR of the 18s rRNA was also done (25).

For RFLP analysis, the PCR product was restricted with RsaI (Toyobo, Japan), while the nested PCR product was digested with SspI (Invitrogen) and VspI (Promega). The PCR and RFLP digested products were fractionated on a 2.0% agarose gel and visualized by ethidium bromide staining using a 2UVTM Transilluminator (Pharmacia). Gel pictures were stored and recorded using Kodak Digital Science software.

DNA sequencing: PCR products of the poly-T locus and the 18S rRNA gene fragments were sequenced by an automated sequencer (AB1310, Applied Biosystems, USA) using the respective primers for the PCR amplifications in the presence of BigDye Terminator Cycle Sequencing Kit (Applied Biosystems).

RESULTS

Sample collection: A total of 3,456 stool samples were collected for this study. Samples came from patients who consulted due to diarrhea in various hospitals and health centers. Collection was done over a 13-month period from May 2004 to May 2005 in all 3 major groups of islands in the Philippines (Fig. 1; Table 1). The highest number (1667, 48.2%) of samples came from Luzon, the largest group of islands in northern Philippines. Visayas is next with 1399 samples (40.5%) and Mindanao in the south had 390 samples (11.3%).

The summary of data on sample collection is given in Table 1. Patients were from <1 to 95 years old with 2160 (63.4%) samples from pediatric (0-18 years old) patients and 1245 (36.6%) from adult (>18 years old). The ratio of male to female patients was 6:4.

Prevalence: From the collection of 3,456 stool samples examined, 133 stools (3.9%) were positive for *Giardia lamblia* and/or *Cryptosporidium* spp. (Table 1). Three (3) positive samples showed co-infection of both *Giardia* and *Cryptosporidium*. Thus, the total number of isolated protozoa was 136. There was no significant difference ($p=0.862$) in the distribution between that of *G. lamblia* (69; 2.0%) and that of *Cryptosporidium* spp. (67; 1.9%).

Out of a total of 133 positive stool samples, 83 (5.0%) were from Luzon, 19 (4.9%) from Mindanao, and 31 (2.2%) from the Visayas. Among the 3 major islands, only Visayas had a significant difference ($p<0.001$) in percentage of positive samples from Luzon and Mindanao. On the other hand, there was no difference ($p=0.93$) in the distribution of positive samples between Mindanao and Luzon.

The overall positivity rate in the Philippines for *Giardia* and *Cryptosporidium* among pediatric patients was 4.8% and in adults it was 2.1%. Among pediatric patients, the prevalence of *G. lamblia* (2.0%) was significantly lower ($p=0.049$) than that of *Cryptosporidium* spp. (2.9%). On the other hand, the prevalence rates of *Cryptosporidium* spp. (0.2%) and *G. lamblia* (1.9%) among adults were significantly different ($p<0.001$).

Between pediatric and adult patients, the prevalence of *G. lamblia* was not significant ($p=0.899$) while the prevalence of *Cryptosporidium* spp. was statistically different ($p<0.001$).

Geographic, age, and sex distribution: The geographic distribution of *Giardia* and

Cryptosporidium is also shown in Table 1. *G. lamblia* was most prevalent in Mindanao ($p=0.050$) while *Cryptosporidium* spp. was most prevalent in Luzon ($p<0.001$).

In Luzon, the prevalence of *Cryptosporidium* spp. (3.1%) was significantly higher than *G. lamblia* (1.9%). However, in Visayas, the prevalence of *G. lamblia* (1.6%) was significantly higher than *Cryptosporidium* spp. (0.6%). There was no sufficient evidence to conclude that the prevalence of *Cryptosporidium* spp. (1.5%) in Mindanao was statistically different from that of *G. lamblia* (3.6%).

Table 2 gives the comparison between the prevalence of *G. lamblia* and *Cryptosporidium* spp. among pediatric and adult patients. Overall, the prevalence of *G. lamblia* among pediatric patients was 1.99%, while it was 1.93% for adults. These rates were not statistically different ($p=0.898$). However, for *Cryptosporidium*, the prevalence (2.92%) among pediatric patients was significantly different ($p<0.001$) compared to that (0.24%) among adults.

Additionally, Table 2 compares the prevalence of *G. lamblia* and *Cryptosporidium* spp. among pediatric and adult patients by sex. Among males, the prevalence of *G. lamblia* and *Cryptosporidium* spp. between pediatric patients and adults were not statistically different ($p>0.05$). On the other hand, the prevalence of the two protozoa among females were significantly higher ($p<0.001$) in pediatric patients than in adults. For pediatric patients, the prevalence of both *G. lamblia* and *Cryptosporidium* spp. was not significantly different ($p>0.05$) between males and females. Among adults, the prevalence of *G. lamblia* but not of *Cryptosporidium* spp. was significantly different ($p=0.004$) between males and females.

Fig. 2 shows the distribution of *G. lamblia* and *Cryptosporidium* spp. by different age groups of pediatric patients. Children less than 4 years old had significantly higher ($p<0.010$) prevalence of *Cryptosporidium* spp. than *G. lamblia*. On the other hand, children aged 4 to 6 years had significantly higher ($p<0.010$) prevalence of *G. lamblia* (7.37%) than *Cryptosporidium* spp. (0.46%). The prevalence rates of the two protozoa were not significantly different in other pediatric age groups.

Seasonality: Seasonality of the occurrence of *G. lamblia* and *Cryptosporidium* spp. among diarrhea patients in the Philippines showed an increasing trend during the rainy season, with a distinct peak in September (Fig. 3). This also coincided with the increasing number of stool samples collected. There seemed to be a higher ($p=0.002$) prevalence of *Cryptosporidium* in the rainy versus dry season (2.59% vs 0.89%) but between the 2 protozoa, there was no difference ($p>0.050$) in prevalence during the rainy or dry season.

Molecular characterization of *Cryptosporidium* spp.: Isolates of *Cryptosporidium* spp. in this study were characterized by PCR-RFLP and sequence analysis of polythreonine and 18S rRNA genes. Out of 67 stool samples (63 from pediatric patients) positive for *Cryptosporidium* oocyst by microscopy, PCR amplification was successful only in 22 samples from pediatric patients.

Table 3 shows that there were 12 C. hominis, 6 C. parvum, 1 co-infection of C. hominis and C. parvum, and 1 C. canis. There were 2 samples, which gave 2 different results. In the first sample, C. hominis was detected by PCR-RFLP of 18S rRNA gene, and C. canis was detected by sequencing of the 18S rRNA gene. In the second sample, C. hominis was detected by PCR-RFLP of the polythreonine gene and by sequencing of the18S rRNA gene, while co-infection of C. hominis and C. parvum was identified by PCR-RFLP of the18S rRNA gene.

In Luzon, where the largest urban center is found, there were 10 C. hominis, 6 C. parvum and 1 co-infection of C. hominis and C. parvum isolates. In Visayas, there were 1 C. hominis, 1 C. canis, and 1 possible co-infection (C. hominis/C. canis). In Mindanao, there were 1 C. hominis and 1 possible co-infection (C. hominis/C. parvum).

DISCUSSION

Over a period of more than 20 years, numerous studies conducted on the prevalence of intestinal parasites have documented the ubiquity of *G. lamblia* and *Cryptosporidium* spp. in the Philippines. Carney and his co-workers (5, 10, 12) conducted surveys on intestinal parasites and found *G. lamblia* to be present in all the areas studied in representative localities in Luzon, Visayas and Mindanao. In a monograph on studies done over a period of 17 years, Cross and Basaca-Sevilla (26) documented information from various surveys on the prevalence of intestinal parasitic infections, including *G. lamblia* and other infectious diseases from all major islands of the Philippines.

In the present study, 3456 stool samples came from all major islands in the Philippines. There was no significant difference between the overall prevalence of *G. lamblia* (2.00%) and that of *Cryptosporidium* spp. (1.94%) in the population of patients with diarrhea included in the study.

In the Philippines, a wide range of prevalence has been reported, depending on the study population.

Studies done in various residential institutions tend to give high prevalence rates for *Cryptosporidium*: 17.6% in mass survey of inmates (13), 11.6% in children of residential institutions in Metro Manila (14), and 9.73% in a mental institution (27). Field surveys had varying results. In a study done in 1973, Cross et al. (4) reported a prevalence of 3% for *G. lamblia* from stool samples collected in Samar Province in the Visayas. Among the urban poor in Metro Manila, Auer (7) found a 20% prevalence of *G. lamblia* in children aged 8 months to 15 years, while Lee et al. (9) obtained 7.8% in children and adolescents in a rural community in the southern part of Luzon. Rivera et al. (28) obtained a significantly low rate of 0.26% in northern Philippines.

At an outpatient clinic at Clark Air Force Base Hospital in Luzon, *Cryptosporidium* lamblia was found in 2% of American military personnel with diarrhea (29). Hospital-based surveys gave surprisingly low rates of 0.6 % in a 2-year survey of etiologic agents of diarrheal disease at San Lazaro Hospital, Manila (6) and 0.4% in a university hospital (8). Carlos et al. (15) reported that among patients with

measles and diarrhea, prevalence of *Cryptosporidium* was 5.7% against controls (with measles without diarrhea) which was 3.1%. A similar study done in KwaZulu-Natal, Africa reported a range of 2.9 - 3.7% for *Cryptosporidium*, and 2.9 - 3.0% for *Cryptosporidium* (30).

The prevalence for *Cryptosporidium* from our nationwide survey is slightly higher than that obtained by Jueco et al. (19) who reported a prevalence of 1.8% in patients of all ages, but lower than 2.6% obtained by Cross (16) among patients aged 1 month to 75 years. While our study is a nationwide survey, the latter studies were done on a limited hospital-based population in Metro Manila.

In a review of over 130,000 diarrhea patients, Adal et al., (31) noted that 6.1% and 2.1% had *Cryptosporidium* infections in developing and in developed areas respectively. However, data show a wide range in the prevalence rates of *Cryptosporidium* in developing countries. At the Siriraj Hospital in Bangkok, Thailand, Thamlikitkul (32) showed an overall prevalence of cryptosporidiosis to be 0.5%. In Korea, Cho et al. (33) reported a positive rate of 22% of 230 out-patients, Chai et al. (34) reported 7.9% from 3146 inhabitants, while Seo et al. (35) reported 1.9% among 461 inhabitants. More recently, Tumwine et al. (36) cite the prevalence to be 3.5% in Turkey, 7.5% in Burkina Faso and 18% in Zambia, while Yu et al. (37) got an overall positive rate of 3.3% in several rural areas in Korea.

Reports on cryptosporidiosis among diarrhea patients in the Philippines are mainly on its prevalence in children. This ranges from 2.5% [0-2 years old] (38), 2.54% [less than 12 years old] (21), 2.9% [6-20 months old] (16), 2.8 % [0-5 years old] (19), 4% [7-19 months old] (18), 7.1% [6-27 months old] (15), to 8.5% [7-24 months old] (17). In this study, the prevalence (2.92%) of Cryptosporidiuim in patients 0-18 years old, is within the range previously reported.

Prevalence rates in two other southeast Asian countries fall within the same range: Myanmar with 3.4% in infants between 2 and 11 months of age (39), and Thailand, 3.7% for children 0-14 years (32).

Our data from the Philippines are consistent with previous reports from developing countries that *Cryptosporidium* occurs more frequently in diarrheic children than in the adult population (32). This trend is expected, since children, especially those below 5 years old, are particularly vulnerable due to high prevalence of malnutrition and poor immune functions that lead to persistent diarrhea. Hunter and Nichols (40) made a review of studies showing that cryptosporidiosis is more common and more severe in malnourished than in well-nourished children. In the Philippines, studies by Paje-Villar et al. (8) and Menorca (20) underscore the role of immune status and malnutrition in cryptosporidiosis among children.

Malnutrition continues to persist in the Philippines despite improvements in primary healthcare. A nationwide survey conducted by Cerdeña et al. in 2001 (41) on 12,425 Filipino children, aged 0-10 years shows that about 6 out of every 100 pre-school age children suffer from acute malnutrition while 31 out of every 100 children are underweight.

Nevertheless, southeast Asia has a better picture than Uganda, where 25.0% of the 1779 children (0-5 years old) with diarrhea had C. parvum (36). Malnutrition, stunting, being underweight, and wasting were significantly associated with the high prevalence of C. parvum. Interestingly, in the same study, the occurrence of C. parvum in a large proportion of diarrheic children less than 3 years of age has been attributed to an early exposure to cryptosporidiosis within a few weeks after birth, which manifest only at 6-24 months of age due to maternal protection through breastfeeding.

Unlike other previous studies done in the Philippines, the present work compares geographical, age and sex distribution of *G. lamblia* and *Cryptosporidium* spp. *G. lamblia* was most prevalent in Mindanao while *Cryptosporidium* was most prevalent in Luzon. Among pediatric patients, the prevalence of *G. lamblia* was significantly lower than the prevalence of *Cryptosporidium* spp. On the other hand, among adults, the prevalence of *Cryptosporidium* spp. was significantly lower than that of *G. lamblia.*

Our present study showed that between pediatric patients and adults with diarrhea, the prevalence of *Cryptosporidium* spp. was statistically different, but not for *G. lamblia.* In the pediatric group, *Cryptosporidium* had the highest prevalence among the 1-3 year olds, while *G. lamblia* was most prevalent among the 4-6 year olds. In the Philippines, Cross (4) and Baldo et al.(14), obtained similar results that show a tendency for *Cryptosporidium* to decrease with age.

Jarmey-Swan et al. (30) studying 2800 children under 5 years in South Africa, indicated that *Cryptosporidium* was most prevalent (39.3%) in the <1 year age group while *Cryptosporidium* was most prevalent in the 3 to 4-year age group (38.5%). Similarly, in our study, the younger children (3 years and below) showed a high prevalence (3.86%) of *Cryptosporidium,* while the older children (4 to 9 years age group) had a high prevalence (6.46%) for *Cryptosporidium.*

Differences in sex distribution for *G. lamblia* and *Cryptosporidium* have no significant impact due to inconsistencies in various reports. In our study, the prevalence of both *G. lamblia* and Crytosporidium spp. for pediatric patients was not significantly different between males and females. On the other hand, Salas (11) showed G. lamblia infection to be higher in males than in females among children 0 to 10 years of age. Among adults, only G. lamblia had a significantly higher prevalence in males compared to females.

In Africa, the same results have been reported for *Cryptosporidium* but not for *Cryptosporidium* (30).

Our study shows that seasonality in occurrence of *Cryptosporidium* and *Cryptosporidium* in the Philippines appears to be correlated with the rainy season, which in turn may also be correlated with the higher incidence of diarrhea during the same period. In their 2-year study, Adkins et al. (6) showed that the number of patients with diarrhea in Manila increased with the onset of the monsoon rains and peaked during the months of maximum rainfall. Similarly, Capeding and Saniel (18) associated cryptosporidiosis in acute diarrhea in children, with episodes predominating during the rainy months of June to September. Salas (11) , working on G.

lamblia infections in Cebu, located in the Visayas, found infection to be low during summer and high during rainy months.

This trend was also seen in Uganda, where the prevalence of *Cryptosporidium* was highest during the rainy months of April to June (36).

On the other hand, Jarmey-Swan et al. (30) did not find any correlation between the incidence of *Cryptosporidium* and *Cryptosporidium* and climactic factors such as rainfall, season or year. It is possible that in the specific study population, personal hygiene, potable water supply, sanitation and education are more significant factors, rather than water-borne transmission.

Preliminary results of our molecular studies show that the predominant species of *Cryptosporidium* in our collection are C. hominis and C. parvum. This information will be useful in establishing mode of transmission as well as in developing preventive measures. Our results are consistent with data from similar studies in other countries (42, 43, 44). As in our study, McLauchlin et al. (45) and Tumwine et al. (43) also reported co-infection by C. hominis and C. parvum in the United Kingdom and Uganda, respectively. The low frequency of C. canis in our study, similar to that in Peru (46), is an indication that zoonotic transmission from dogs is not common in the Philippines.

We obtained an interesting result from a patient whose sample had 2 different results. Using PCR-RFLP of the 18S rRNA gene, C. hominis was detected. However, by sequencing of the same gene, which was amplified from a different DNA preparation, C. canis was identified. Our conclusion in this case is that the patient had co-infection. As previously suggested (47), amplification of a single species by PCR is not a conclusive indication that the sample contains only one species. In the present study, the possibility of co-infection by C. hominis and C. parvum is most likely because different batches of extracted DNA were used separately to amplify a segment of the *Cryptosporidium* DNA.

In the present study, data on molecular characterization are limited due to the big number (45/67, 67%) of samples with failed PCR amplification. One reason is the storage of samples in buffered formalin, which may interfere with cell lysis, as well as inhibit DNA amplification, (48). In addition, according to Clark (49), some components in the stool could also inhibit or reduce the sensitivity of PCR. In the present study, 22 out of 67 (33%) *Cryptosporidium* isolates were successfully genotyped. In our ongoing molecular study on enteric protozoa, we extract DNA only from fresh stool samples and our results indicate a significant improvement in the success rate of PCR amplification.

Other workers have attributed failed PCR of microscopy-positive samples to various factors. Xiao et al. (46) who had a success rate of 49%, explained that failed PCR was due to accidental freeze-thawing of samples several times during storage. McLauchlin and co-workers (50) had a significantly higher success rate of 97%. They gave the following reasons for failed PCR: possible extraction of no or insufficient amount of DNA, presence of no specific target DNA, or presence of other organisms

which gave false positive results for microscopy.

TABLES AND FIGURES

Table 1. Summary of data on sample collection and microscopy.

Demographic Profile	Stool Samples (#/%)	Prevalence (frequency/percentage)		
		Total positive samples	*Cryptosporidium*	*Cryptosporidium*
Site of collection				
Luzon**	1667 (48.2)	83 (4.98)	32 (1.92)	52 (3.12)*
Visayas**	1399 (40.5)	31 (2.22)*	23 (1.64)	9 (0.64)
Mindanao	390 (11.3)	19 (4.87)	14 (3.59)*	6 (1.54)
Overall (Philippines)	3456 (100.0)	133 (3.85)a	69 (2.00)	67 (1.94)
Subject classification				
Pediatric** (0-18 years old)	2160 (63.4)	104 (4.81)	43 (1.99)	63 (2.92)*
Adult** (>18 years old)	1245 (36.6)	26 (2.09)	24 (1.93)	3 (0.24)
Total	3405 (100.0)b	130 (3.82)c	67 (1.97)	66 (1.94)
Sex				
Male	1934 (56.0)	79 (4.08)	42 (2.17)	39 (2.02)
Female	1520 (44.0)	54 (3.55)	27 (1.78)	28 (1.84)
Total	3454 (100.0)d	133 (3.85)e	69 (2.00)	67 (1.94)

* Significantly different (site of collection; subject classification)

** Significantly different (*Cryptosporidium* vs. *Cryptosporidium*)

a Three (3) patients had co-infection.

b Fifty-one patients with unknown ages were excluded from the analysis.

c Three (3) patients with unknown ages were excluded from the analysis; 3 patients had co-infection.

d Two patients with unknown sex were excluded from the analysis.

e Three (3) patients had co-infection.

Table 2. Prevalence of G. lamblia and *Cryptosporidium* spp. among pediatric and adult patients by sex.

Sex	*Cryptosporidium* lamblia		*Cryptosporidium* spp.	
	Pediatric (N=2160) Freq. (%)	Adult (N=1245) Freq. (%)	Pediatric (N=2160) Freq. (%)	Adult (N=1245) Freq. (%)
Male	23 (1.06)	19 (1.53)	36 (1.67)	2 (0.16)
Female	20 (0.93)*	5 (0.40)	27 (1.25)*	1 (0.08)
Total	43 (1.99)	24 (1.93)	63 (2.92)*	3 (0.24)

* Significantly different (pedia vs. adult)

Table 3. Isolates and genotypes of *Cryptosporidium* spp. from pediatric patients in the Philippines

Isolate code	Location	Polythreonine gene		18S rRNA gene	
		RFLP	Sequencing	RFLP	Sequencing
NCR 038	Luzon		*C. parvum*		
NCR 044	Luzon	*C. hominis*	*C. hominis*		
NCR 060	Luzon		*C. hominis*		
NCR 070	Luzon	*C. hominis*	*C. hominis*		
NCR 076	Luzon		*C. hominis*		
NCR 111	Luzon	*C. hominis*	*C. hominis*		
NCR 134	Luzon	*C. hominis*	*C. hominis*		
NCR 192	Luzon	*C. hominis* & *C. parvum*	*C. hominis* & *C. parvum*		
NCR 234	Luzon	*C. hominis*	*C. hominis*	*C. hominis*	
NCR 306	Luzon			*C. parvum*	
NCR 320	Luzon			*C. parvum*	
NCR 332	Luzon			*C. parvum*	
NCR 398	Luzon	*C. parvum*			
NCR 826	Luzon			*C. hominis*	
NCR 887	Luzon			*C. hominis*	
LUZ 272	Luzon	*C. hominis*		*C. hominis*	
LUZ 419	Luzon			*C. parvum*	
VIS 046	Visayas				*C. canis*
VIS 152	Visayas			*C. hominis*	*C. canis*
VIS 682	Visayas			*C. hominis*	
MIN 016	Mindanao	*C. hominis*		*C. hominis* & *C. parvum*	*C. hominis*
MIN 176	Mindanao	*C. hominis*			