図2 CPGP40/15遺伝子一部領域の系統樹解析

従来の遺伝子型別はヒト型（I）並びにウシ型（II）に大別するものであったが、図では
CPGP40/15領域での系統樹解析により、それぞれが複数のクラスターを形成しており、
より詳細な分別が可能であった。本研究で決定した配列（Nagano）はIfに属することが
示された。

Philippine Report on

Prevalence and Genotypic Diversity
of Enteric Protozoa Isolated from the Philippines

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Prevalence and Genotypic Diversity of Enteric Protozoa (i.e. *Giardia* and *Cryptosporidium)* Isolated from the Philippines

INTRODUCTION

Diarrheal diseases continue to be an important public health problem in developing countries. In the Philippines, diarrhea was the 2nd leading cause of morbidity in 2002 and the 1st in 2001 (Appendix 1). The most common etiologic agent that causes diarrhea is the Rotavirus (50% of cases) followed by enterotoxigenic *Escherichia coli* (ETEC) and other bacterial agents such as *Campylobacter, Shigella, Vibrio cholera* and non-typhoidal *Salmonella*. Around 25% of diarrhea cases have unidentified etiologic agents.

The enteric protozoa *Giardia lamblia* and *Cyclosporidium* spp. have been recognized as emerging pathogens, which cause diarrhea in both children and adults. They have also been implicated as common intestinal parasites of a wide range of vertebrates. In the Philippines, the lack of surveillance data and limited diagnostic facilities in primary healthcare centers have led to failure to recognize the importance of these parasitic protozoa as public health hazards.

In a nationwide survey on diarrheic patients in the Philippines conducted by the St. Luke's Medical Center Enteric Protozoa Research Network (Appendix 2), enteric protozoa were identified from stool samples using standard microscopy and immunofluorescence techniques. From 3545 stool samples, *Entamoeba* spp., *Giardia lamblia, Cryptosporidium* spp., *Cyclospora* sp. and *Isospora* sp. were identified.

Microscopic examination of stool samples is the accepted gold standard method for detection of intestinal pathogens. In the case of enteric protozoa, the use of dyes and staining techniques has been used as aid in identification. Rapid detection tests using fluorescent microscopy have been developed commercially. These tests are very useful for practical diagnostic purposes in hospitals and clinics. However, there is a disadvantage in the use of these commercial kits, as they are able to detect only a narrow range of protozoa that are considered most prevalent.

Molecular techniques such as PCR, RFLP, and sequencing, have proven to be reliable as a direct method for characterization of protozoa from fecal samples. Due to the fastidious nature of these pathogens, such tools offer a faster and more sensitive identification method, as well as a better alternative to culture. Furthermore, they allow identification of genetic markers that can differentiate various isolates, which is not possible by conventional microscopy.

In this study, molecular characterization was done on *Cryptosporidium* sp. isolated from stool samples. Preliminary results presented in this report include species identification and data on genetic variability of Philippine *Cryptoporidium* isolates.

Such information may be useful in making correlations between genotypes and pathogenicity, and in understanding host response and disease severity. This is the first report on the molecular diversity of these parasitic protozoa from the Philippines.

METHODOLOGY

Patient Information

Patients included in the study were asked to provide their personal data and background information related to the diarrhea as well as clinical symptoms.

All Patient Information Sheets were collated and encoded in a database. The information was encoded using Microsoft Access.

Field collection

Collection of stool samples was done by following the instruction for field collection, i.e. 1ml of stool sample from a diarrheic patient was added into a polypropylene tube containing 9 ml of formalin. Samples collected from the field were brought for laboratory processing at the Research and Biotechnology Division, St. Luke's Medical Center. All samples were coded according to collection site, date of collection, and stored at 4°C until further processing.

Samples

Collection of stool samples

Samples used for the study are from a previous collection of stools from diarrhea patients in the Philippines. The samples were collected from May 2004 to June 2005 in all main islands namely Luzon. Visayas, and Mindanao.

One ml of stool sample from a diarrheic patient was placed in a 15 ml polypropylene tube containing 9ml of formalin solution. The samples were stored at 4°C until these were sent to St. Luke's Medical Center for processing.

Processing of stool samples

Tubes containing 9ml of 10% formalin and 1ml stool sample were vortexed and filtered. After adding 2ml of ethyl acetate, the filtrate was mixed vigorously and then centrifuged at 1000 x g for 5 minutes at 4°C.

The pellet was then washed with 2ml of 0.1% Tween20, centrifuged and the supernatant removed. PBS was added to the pellet for a final volume of 50μl.

Microscopy

Detection of Giardia_and_Cryptosporidium using the Merifluor® Kit

Five ul of the processed sample was dropped on a slide, stained with Merifluor® kit and observed under a fluorescence microscope with a blue excitation filter. Photomicrographs were taken immediately for documentation.

DNA Analysis

After microscopy, samples that showed the presence of *Giardia* or *Cryptosporidium* were stored separately to be used for future DNA studies. Only *Cryptosporidium* spp.are included in this report. Analysis of *Giardia* will be done on the second year.

Extraction of Genomic DNA (QIAamp DNA Stool Minikit)

Genomic DNA was extracted from *Giardia* and *Cryptosporidium* isolates using optimized DNA extraction protocols.

Analysis of *Cryptosporidium* spp. from the Philippines

Attempts were done to isolate DNA from formalin-fixed stool samples.

DNA isolated was used for genotyping of *Cryptosporidium* spp. isolates from the Philippines using 18s SSU rRNA, and/or polythreonine gene.

PCR was performed to amplify specific regions of 18s SSU rRNA, and/or the polythreonine gene. For the polythreonine gene PCR product, RFLP was done using *RsaI*. The 18s SSU rRNA PCR product was digested with *SspI* and *VspI*, and sequenced for genotyping. Multiple sequence alignment using BLAST was performed for species identification.

RESULTS AND DISCUSSION

The number of stool samples collected during the period May 2004 to June 2005 is 3545. The geographical distribution of these samples is shown in Table 1. The highest collection is from Luzon, the norhernmost and biggest group of islands in the Philippines, followed by Visayas, the middle group of islands. As to age distribution, almost two-thirds (62.4%) of the samples came from pediatric patients (Table 2). This is consistent with data from the National Statistics Office that prevalence of diarrhea in children is higher than in adults.

Table 1. Distribution of samples in three major islands in the Philippines.

| Collection area	Frequency	Percentage
Luzon	1701	48.0
Visayas	1445	40.8
Mindanao	399	11.3
Total	3545	100

Table 2. Age distribution of diarrhea patients included in this study

Subject classification	Frequency / Percentage
Pediatric (0-18 years)	2212 (62.4)
Adult (>18 years)	1278 (36.1)
SubTotal	3490 (98.4)
No information	55 (1.6)
Total	3545 (100.0)

This report focuses on the detection of *Giardia* cysts and *Cryptosporidium* oocysts from the stool collection, although other enteric protozoa were identified as well. The MeriFluor® kit could distinguish cysts of *Giardia* from oocysts of *Cryptosporidium* morphologically. While both exhibit fluorescence with blue light excitation, *Giardia* cysts are much bigger than oocysts of *Cryptosporidium* (Figures 1 and 2). Our isolates showed the typical oval shape of *Giardia* cysts, with sizes ranging from 7 x 11 μm to 11 x 14 μm. *Cryptosporidium* oocysts are spherical, and ranged from 4.30 x 5.30 μm to 5.70 x 6.30 μm. The other morphological features include the axonemes and nuclei for *Giardia* and the four sporocysts in *Cryptosporidium*.

Table 3 shows the prevalence of *Giardia* and of *Cryptosporidium* as determined from this current collection. In this study, the overall frequency of *Giardia* and *Cryptosporidium* in the Philippines was 3.9%. There is no significant difference in the prevalence of *Giardia* (2.0%) and that of *Cryptosporidium* (1.9%).
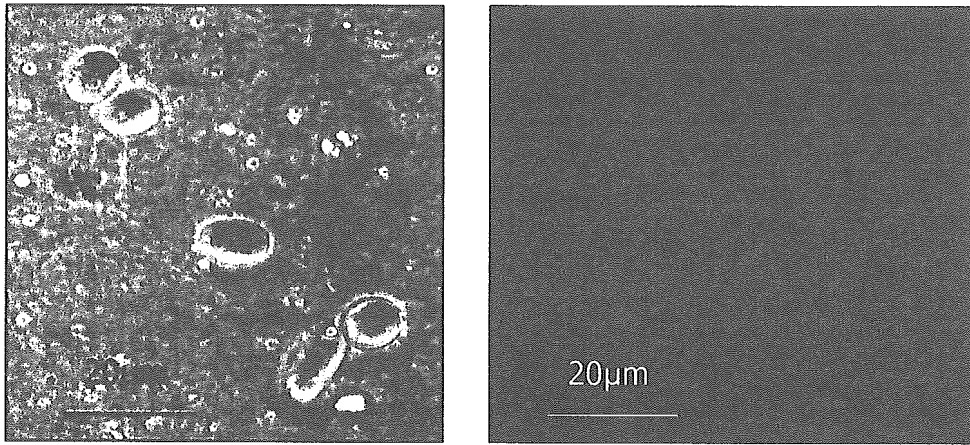
Figure 1. *Giardia lamblia* cysts
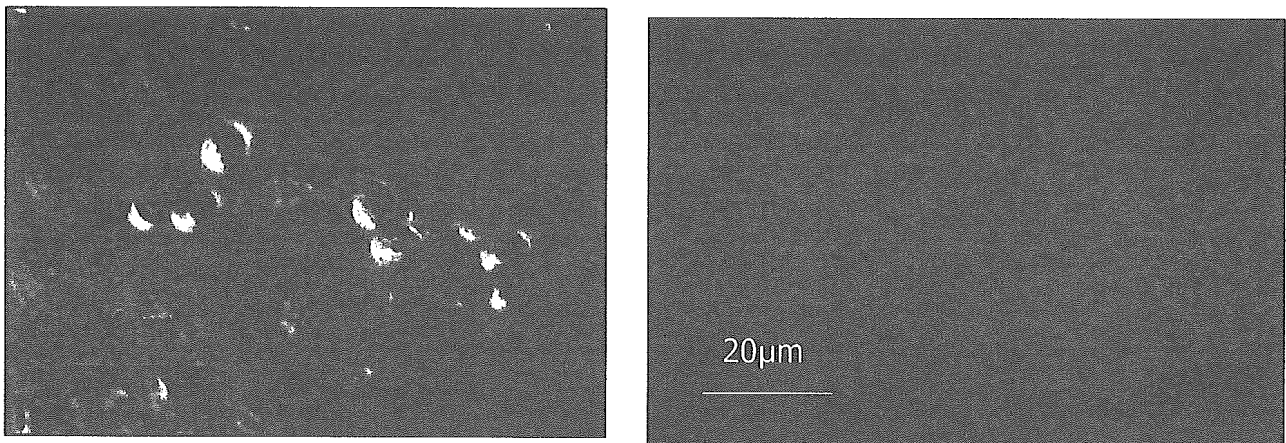
Figure 2. *Cryptosporidium* spp. oocysts

Table 3. Prevalence of Enteric Protozoa (*Giardia* and *Cryptosporidium*)	
Protozoa	**Frequency / Percentage (n=3545)**
Giardia	71 (2.0)
Cryptosporidium	67 (1.9)
Total	**135 (3.9)**

However, with respect to geographic distribution (Table 4), the frequencies of *Giardia* and *Cryptosporidium* vary in the three islands.

Giardia was found to have the highest frequency in Mindanao, the southernmost group of islands in the Philippines. The frequencies in Luzon and Visayas were 50% lower. This may be explained by the fact that Mindanao is primarily an agricultural area, where drinking water comes from underground sources. Thus, re-infection may be common.

The highest prevalence for *Cryptosporidium* was found in Luzon with a frequency of 3.1%. This may reflect the poor sanitation that prevails in the depressed communities of Metro Manila, which comprise 40% of the Philippines' urban poor. Most of the Luzon samples were collected from residents of depressed areas, characterized by lack of clean and adequate source of water.

Table 4. Geographic distribution of *Giardia* and *Cryptosporidium* isolates		
	Giardia Frequency / Percentage	*Cryptosporidium* Frequency / Percentage
Luzon	33 (1.9)	52 (3.1)
Visayas	24 (1.7)	9 (0.6)
Mindanao	14 (3.5)	6 (1.5)
Overall (Philippines)	**71 (2.0)**	**67 (1.9)**

Molecular characterization of *Cryptosporidium* was done by genotyping using RFLP of 18s SSU rRNA and polythreonine gene. As shown in Table 5, human (*C. hominis*), bovine

(*C. parvum*), and canine (*C. canis*) genotypes were isolated from the Philippines. The majority (10 out of 15) was the human genotype, indicating that anthroponotic transmission is common. The presence of the bovine and canine genotypes provides evidence of the occurrence of zoonotic transmission as well.

Table 5. Results of Genotyping of Philippine *Cryptosporidium* Isolates	
Genotypes	**No. of Isolates (n=15)**
Human genotype (*C. hominis*)	10
Bovine genotype (*C. parvum*)	2
Mixed genotypes (*C. hominis* and *C. parvum)*	1
Canine genotype (*C. canis*)	2
TOTAL	**15**

Table 6 shows that the genotypes in Luzon tend to be more diverse, as compared to the other islands. However, no conclusion can be made at this point since these are only our partial molecular data.

Table 6. Geographic distribution of *Cryptosporidium* spp.			
***Cryptosporidium* species**	**LUZON**	**VISAYAS**	**MINDANAO**
C. hominis (Human genotype)	8	0	2
C. parvum (bovine genotype)	2	0	0
C. hominis and *C. parvum* (mixed human and bovine genotype)	1	0	0
C. canis (canine genotype)	0	2	0
TOTAL	**11**	**2**	**2**

Based on demographic data, all 15 samples with genotyping data were from pediatric patients aged 3 months to 3 years. Fifty-three percent were males. There were no correlations found between cryptosporidiosis and stunted, underweight, or obese patients. Weight loss was statistically significant in 2 out of 15 patients. Among the 15 patients, none was immunocompromised.

PROBLEMS and RECOMMENDATIONS

Problems	Recommendations
1. Sample Collection and Transport	
• Delays due to bureaucratic procedures needed for collaboration	• Select institutions that do not require formal agreements.
• Transport of samples from remote areas still difficult	• Identify local couriers accessible within the vicinity
2. Laboratory work	
• Formalin-fixed samples are difficult to use for DNA isolation	• Perform other fixation protocols (Note: Trial on EtOH fixation to overcome difficulty is now ongoing)
• DNA extraction procedures are still to be optimized	• Consultation with other members of the network should be done to standardize protocols.
• Lack of expertise of laboratory staff	• Training-workshop for laboratory staff.
3. Data collection	
• Incompletely filled-up questionnaires	• Reminders to all collaborating institutions regarding the importance of data collection.
• Availability of personnel from collaborating institutions to collect data	• Give incentives to personnel

ACKNOWLEDGEMENT

APPENDICES

Appendix 1. Ten Leading Causes of Morbidity in the Philippines, 2001-2002*

	2001	Disease	2002
Diarrheas	845,526	Pneumonias	734,581
Bronchitis/Bronchiolitis	694,836	Diarrheas	726,310
Pneumonias	652,585	Bronchitis/Bronchiolitis	629,968
Influenza	499,887	Influenza	484,388
Hypertension	318,521	Hypertension	304,690
TB Respiratory	110,841	TB Respiratory	114,221
Diseases of the Heart	47,040	Diseases of the Heart	52,237
Malaria	40,543	Malaria	39,994
Measles	24,494	Chickenpox	28,600
Chickenpox	24,359	Measles	24,639

*http://www.doh.gov.ph/data_stat/html/healthstatistic.htm

Appendix 2. The Philippine Enteric Protozoa Network

In May 2004, the Research and Biotechnology Division of St. Luke's Medical Center took the lead in forming the Philippine Enteric Protozoa Network. The network was formed primarily for the field collection of stool samples from diarrhea patients. Sites were selected from all 3 major islands of the Philippines and letters were sent to various institutions and possible collaborators. Meetings were held with those who responded positively to the invitation.

The meetings were usually attended by hospital administrators, physicians in the area, pathologists, and nurses. In some cases, laboratory supervisors or medical technologists were also invited. All meetings were successful in establishing a network of collection sites. The agenda of the meetings included a discussion of the objectives of the study and the general protocol, and an orientation on the collection, storage, and transport of stool specimens. Emphasis was given on the importance of the completion of the patient information sheet. Participants were also assured that they will have access to the data pertinent to the samples from their sites. They were also assured that all expenses relative to their participation in the study will be shouldered by the project.

Some of the issues brought up by the participants included the following:

- Review of Ethics Committees of participating hospitals
- Inclusion and exclusion criteria
- Duration of collection period
- Duration of project
- Incentives for project personnel
- Training program that can be availed by hospital personnel
- Lack of storage space
- Shipment of samples
- Scope of patient recruitment
- Few samples from a center may not be good enough for a research paper
- Lack of manpower to be assigned to the project
- Patients have been managed by the time they are referred to the hospital

Communication with members of the network is maintained up to this time.

Appendix 3.   Project Staff

Overall Coordinator

Filipinas F. Natividad, PhD

Project Leaders

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Blanquita B. de Guzman, PhD
Corazon C. Buerano, PhD
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Support Staff
LorilieA. Nibal
Jorge D. Bonganay

Main Project: Molecular characterization of *Cryptosporidium* spp, *Isospora belli*, *Giardia intestinalis* and *Blastocystis hominis* among Thai patients

Project 1: First Year Report

Title: Molecular epidemiology of cryptosporidiosis among human immunodeficiency virus-infected patients in Thailand: analysis of the 18S RNA and the Cpg60/45/15 loci

Name of Researchers: Somchai Jongwutiwes   MD, PhD [1]

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Summary:

Diarrheal disease caused by *Cryptosporidium* sp. is globally prevalent, affecting both immunocompetent and immunocompromised hosts. The consequences of cryptosporidiosis in human immunodeficiency virus (HIV)-infected patients can lead to more significant morbidity and mortality than individuals with normal immune status. Because an effective anti-cryptosporidial agent is currently unavailable, prevention of disease transmission is of primary importance. Several epidemiological studies indicate that human cryptosporidiosis is caused by diverse species of *Cryptosporidium*, one of which deploys exclusive anthroponotic transmission cycle and others are considered zoonosis. To examine the extent of species of *Cryptosporidium* infecting humans in Thailand, we recruited 67 isolates from HIV-infected patients who sought medical treatment at King Chulalongkorn Memorial Hospital in Bangkok during 1997-2003. Determination of species was performed by analysis of partial sequences of the 18S RNA gene encompassing species-specific

domain and compared with those in the GenBank database. The Cpg60/45/15 gene was used for subgenotypic analysis of *Cryptosporidium*. After PCR amplification and purification, sequences were determined directly from the PCR-amplified products and/or from subclones which were done from both directions. Results revealed that the 18S RNA genes defined 8 sequence types belonging to *C. hominis* (38.6%), *C. meleagridis*, (28.6%), *C. parvum*, (12.9%), *C. canis*, (10%), *C. felis*, (2.8%), *C. muris*, (1.4%), *Cryptosporidium* Pig1 genotype (1.4%), and a novel sequence (4.3%). Mixed infections between *C. hominis* and *C. meleagridis* were observed in 3 isolates (4.3%). Phylogenetic construction has placed the novel sequence type of the 18S RNA gene close to that of *C.parvum*, kangaroo genotype. Meanwhile, PCR amplification targeting the Cpg65/45/15 gene of *Cryptosporidium* has been successful for *C. hominis*, *C. parvum* and *C. meleagridis* while the rest yielded negative results. The Cpg64/45/15 sequences obtained from *C. hominis* belonged to groups Ia, Id and Ie while those from *C. parvum* displayed a novel type II sequence and all *C. meleagridis* in this study possessed type IIIa sequence. Sequence comparison with those previously reported has shown that the Cpg65/45/15 gene was highly polymorphic, rendering it an attractive marker for subgenotyping or strain differentiation of *C. hominis*, *C. parvum* and *C. meleagridis*. In addition, no significant correlation between species or subgenotypes of *Cryptosporidium* and clinical symptoms of the patients was found in this analysis. Hence, human acquisition of *Cryptosporidium* can plausibly be from diverse sources and the zoonotic transmission cycles of this coccidian protozoan in Thailand are common among HIV-infected patients.

Introduction

    Cryptosporidium, coccidian protozoa in phylum Apicomplexa, is responsible for significant enteric morbidity in both immunocompetent and immunocompromised individuals. Human infection with *Cryptosporidium* spp. can occur as sporadic cases or large-scale outbreaks affecting several hundred thousand people. Despite a higher prevalence of human cryptosporidiosis in developing than developed countries, its impact on public health is of considerable importance. Clinical presentations of human cryptosporidiosis range from asymptomatic infections to chronic and life-threatening diarrhea depending on the immune status of the hosts. Chronic diarrheal illness due to cryptosporidiosis in human immunodeficiency virus-1 (HIV-1)-infected patients has been attributed to remarkable decreased in quality of life (1,2).

    Besides 2 commonly identified species of *Cryptosporidium*, namely *C. hominis* that occurs exclusively in humans and *C. parvum* that undergoes both anthroponotic and zoonotic transmission

cycles, a number of zoonotic species of *Cryptosporidium* have been implicated in human infections. However, the distribution of species of *Cryptosporidium* found in humans shows geographic variation, probably depending on endemicity of certain species or different sources of infections. Although a number of antimicrobial agents have been evaluated for therapeutic efficacy against cryptosporidiosis, to date no such agent exhibits consistently effective anti-cryptosporidial activity. Hence, understanding the modes and potential sources of disease transmission is of prime importance for appropriate preventive and control measures. In this context, differentiation of species and strain of *Cryptosporidium* spp. responsible for infections or outbreaks needs to be performed (3,4).

Meanwhile, a mucin-like glycoprotein designated gp60/45/15 has been recently identified on the zoite surface of *C. parvum*. The precursor of gp60/45/15 is expressed during merogony as a 60 kDa glycoprotein and subsequently subject to proteolytically process to 15- and 40-45 kDa frgments. Antibodies specific to gp15/45 are capable of neutralizing infection in vitro, suggesting potential roles in attachment to, and invasion of host cells (5-8). Molecular analysis of the gene encoding gp40/15 reveals extensive sequence diversity among clinical isolates, rendering it a useful marker for subgenotyping *C. parvum* and *C. hominis*.

Recently, we have identified at least 5 species of *Cryptosporidium* circulating in Thai patients who had underlying HIV-1 infection and posssesed low CD4 lymphocyte count (9). To further address if more species of *Cryptosporidium* could be the causative agents of human cryptosporidiosis in Thailand, we recruited additional samples for species identification based on the small subunit ribosomal DNA sequences and strain differentiation by the Cpg60/45/15 sequences.

Purposes:

1. To determine species of *Cryptosporidium* among Thai patients based on the 18S rRNA sequences.
2. To analyze subspecific variation in human isolates of *Cryptosporidium* found in Thailand.

Methods:

Cryptosporidium isolates

Stool samples from HIV-infected patients who had diarrhea were screened for the presence of *Cryptosporidium* oocysts by modified kinyoun acid-fast methods at parasitology laboratory of King Chulalongkorn Hospital in Bangkok during 1996-2002. Background demographic, clinical and laboratory data were retrieved for analysis. Positive samples were preserved by adding 3 volumes of

absolute ethanol to one volume of fecal sample and stored at ambient temperature until study (10). Morphometric measurement of at least 20 oocysts per isolate was performed as described. 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 *Cryptosporidium* oocysts were done by the method previously described except that QIAamp DNA stool mini kits (QIAGEN, Germany) were used to extract DNA from sediment of the samples.

Amplifications of *SSU rDNA* and *Cpg60/45/15*

Amplification of the SSU rDNA fragment was performed by nested polymerase chain reaction (nested PCR) as described using outer primers CR-P1 and CR-P2 and inner primers CR-P3 and CPB-DIAGR. The complete cpg60/45/15 gene was amplified by 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), *Cryptosporidium* DNA and 0.2 mM of each primer. The forward primer was CPG40F: 5'- CCCTTCTATAGGTGATAATTAGTCAG-3'and the reverse primer CPG40R: 5'-GTAAAAACTGGCGTCCTGATTCAATG-3'which span the full open reading frame of Cpg60/45/15. The thermal cycler profile contained 35 cycles of 94, 55 and 72°C for 30, 40 and 120 s, respectively. Semi-nested PCR using primers CPG-F1: 5'-GTAGGCAACTAAGGACAAAGG-3' and CPG-R and the same amplification conditions were performed for isolates in which PCR products were not obtained using the first pair of primers. The PCR amplified products were analyzed by electrophoresis on 1 and 2% agarose gels for SSU rDNA and Cpg60/45/15, respectively, 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. Phylogenetic

tree was performed as implemented in the MEGA program (11).

Results

Species of *Cryptosporidium* found in Thai HIV-infected patients

The 18S RNA genes defined 8 sequence types of *Cryptosporidium* in Thai HIV-infected patients, belonging to *C. hominis* (38.6%), *C. meleagridis,* (28.6%), *C. parvum,* (12.9%), *C. canis,* (10%), *C. felis,* (2.8%), *C. muris,* (1.4%), *Cryptosporidium* Pig1 genotype (1.4%), and a novel sequence (4.3%). Mixed infections between *C. hominis* and *C. meleagridis* were observed in 3 isolates (4.3%) (Table 1). Phylogenetic construction has placed the novel sequence type of the 18S RNA gene close to that of *C. parvum*, kangaroo genotype (Figure 1). Analysis of clinical profile of cryptosporidiosis patients failed to detect discernible correlation between disease severity in terms of duration of diarrheal symptoms and frequency of daily diarrheal episodes (data not shown).

Subspecies characterization of *Cryptosporidium* based on the Cpg60/45/15 gene

To date, 6 major sequence types of the Cpg60/45/15 gene of *C. hominis* have been reported (5,12). Of these, 5 types were identified in this study and the most prevalent species was genotype Ia, followed by genotypes Ie, Id, If and Ib, respectively. Two distinct Cpg60/45/15 genotypes were detected among 9 isolates of *C. parvum*, one of which has been newly found in this analysis. Among 20 isolates of *C. meleagridis*, 3 genotypes of the Cpg60/45/15 locus were observed among Thai isolates (Figure 2).

Discussions

Our previous analysis of *Cryptosporidium* species using limited number of samples has shown that *C. hominis* was the most prevalent species found in adult HIV-infected patients while zoonotic species of *Cryptosporidium* were mainly identified in children (13). The present report has recruited more isolates for analysis and it is not unexpected that more species including a novel sequence type was detected than our previous study. The presence of high prevalence of *C. hominis* in Thai patients has implied that transmission via fecal-oral contamination could be the major route for disease transmission because this species is confined to human host. However, zoonotic species or strains, i.e. *C. meleagridis, C. parvum, C. felis, C. muris, C. canis* and *C. parvum* (pig genotype), remains important ones.

The distribution of *Cryptosporidium* species seems to show geographical variation (Figure 3). Although the number of samples for species identification varied from each study, *C. parvum* is the