Figures and Tables

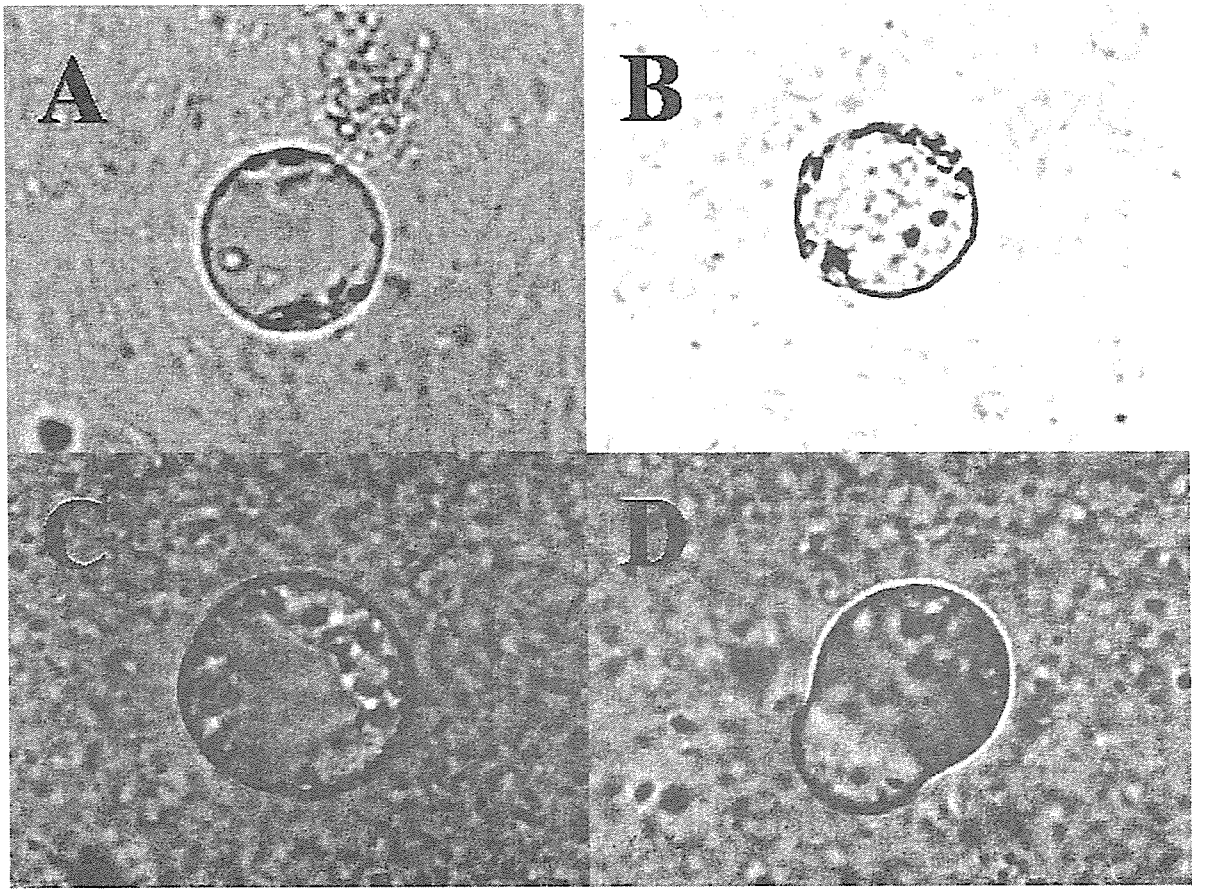

Figure 1 *In vitro* growth of *Blastocystis* in Boeck and Drbohlav's medium containing Locke's solution and egg slant. A-D depict vacuolar, granular, multivacuolar and amoeboid forms, respectively.

```
#Subtype_I   AGTAGTCATA CGCTCGTCTC AAAGATTAAG CCATGCATGT GTAAGTGTAA ATATAGT-AG TTTGGAACTG CGAATGGCTC ATTATATCAG TTATAGTTTA
[ 100]
#Subtype_III ..........  ..........  ..........  ..........  ......A...  ...GTTA-.C  ....A.....  ..........  ..........  ..........
[ 100]
#Subtype_II  ..........  ..........  ..........  ..........  ......C...  ...GTT.T..  .G..A.....  ..........  ..........  ..........
[ 100]
#Subtype_V   ..........  ..........  ..........  ..........  ......A...  C..ATTG-.C  ....A.....  ..........  ..........  ..........
[ 100]

#Subtype_I   TTTGGTGAAG TGTACTACTT GGATAACCGT AGTAATTCTA GGGCTAATAC ATGAGA---- ------AAGT CCTCTGGTGA GGTGTGTTTA TTAGAATGAA
[ 200]
#Subtype_III ....A.....  AA.....A..  ..........  ..........  .A........  ...TAT----  ------..AG  T..T--..AG  AC..CA....  ..........
[ 200]
#Subtype_II  .....AAT..  .-.T...TA.  ..........  ..........  ..........  ...T.TTTTC  TTTGGTGG.C  AAC.ATTAA.  .AA..AC...  .....CAT..
[ 200]
#Subtype_V   ..........  GA.....A..  ..........  ..........  ..........  .....T----  ------..AG  T.C.-.T..G  .A........  ..........
[ 200]

#Subtype_I   AACCATATGC TTCGGCATGA TAGTGAGTAA TAGTAACCT- --ATCGTATC GCATGCTTAA T-GTAGCGAT GAGTCTTTCA AGTTTCTGCC CTATCAGCTT
[ 300]
#Subtype_III .......G.T  ......C.AT  .C........  ..A....TA-  --...A....  .T.......-  .-...A....  .T........  ..........  ..........
[ 300]
#Subtype_II  .....A----  ..TAATG.TG  .T........  ..A...TTGT  TG.CTT....  ........GT  .A........  .TTC......  ..........  ..........
[ 300]
#Subtype_V   ..........  ..........  ..........  .........-  --........  ..........  .-........  ..........  ..........  ..........
[ 300]

#Subtype_I   TCGATGGTAG TATATGGGCC TACCATGGCA GTAACGGGTA ACGAAGAATT TGGGTTCGAT TTCGGAGAGG GAGCCTGAGA GATGGCTACC ACATCCAAGG
[ 400]
#Subtype_III ..........  .G...T..A.  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
[ 400]
#Subtype_II  ..........  .G...T..A.  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
[ 400]
#Subtype_V   ..........  C.......T.  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
[ 400]

#Subtype_I   AAGGCAGCAG GCGCGTAAAT TACCCAATCC TGACACAGGG AGGTAGTGAC AATAAATCAC AATGCGGGAC TATACGTCTT GCAATTGGAT TGAGAACAAT
[ 500]
#Subtype_III ..........  ..........  ..........  ..........  ..........  ..........  .......A..  A..--..T..  ..........  .........C
[ 500]
#Subtype_II  ..........  ..........  ..........  ..........  ..........  ........T.  ...A..AAG.  CT.TG..T..  .T.....A..  ..........
[ 500]
#Subtype_V   ..........  ..........  ..........  ..........  ..........  ..........  ..........  .G.TA.....  ..........  .........C
```

```
[ 500]

#Subtype_I   GTACAACTCT TATCGATAAG CCATTGGAGG GCAAGTCTGG TGCCAGCAGC CGCGGTAATT CCAGCTCCAA TAGCGTATAT TAACGTTGTT GCAGTTAAAA
[ 600]
#Subtype_III ......AC.. .........A .A..... ..... ..... ..... ..... ..... ..... .....
[ 600]
#Subtype_II  ......TCG. .........A .A..... ..... ..... ..... ..... ..... ..... .....
[ 600]
#Subtype_V   ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
[ 600]

#Subtype_I   AGCTCGTAGT TGAAGTGTGG GTGATCGCTG TTGTGAG--- --ACTTCGGT CTCTCGATAG TAAGTCACCC CCT--TCCAG TATCCAGT-- AGTGGG-TAT
[ 700]
#Subtype_III ..... ....A..AA. .GTGGTTG.. .AA...AT-- AC.T.CGT.. A.T.T.T..T .CTAC..... T.C--.T.TA A..T.GA.AT .-..A.-...
[ 700]
#Subtype_II  ..... ......T.A. A.A..TTA.T GAAA.T.TTT GT.T.CGTAC A.TCTAC.TT ..T..A.GTT ATCGACATCC AGGTGG..GC CA...TTCCG
[ 700]
#Subtype_V   ..... .......A.. .A.TGT.TGT .CA....--- --.GC.A.-- -......GT. .TCACA...T ..C--C..TC C.AT.G..GT ......G...
[ 700]

#Subtype_I   TCAGTTACT- TACTACTGTG TGTTGGTCCT TTACTGTGAG AAAATTAGAG TGTTCAAAGC AGGCGTTT-- ---GCTTGAA TAGATTAGCA TGGAATAATA
[ 800]
#Subtype_III .A.T.....T GTA..TG..T .--.A.A... ..... ..... ..... ....A...-- ---..... ..T..... .....C
[ 800]
#Subtype_II  CA..GA.T.G .GG....AGA C--.....A. ..... ..... ....T..... G.A.T...AT TCA.T..... ..... .....
[ 800]
#Subtype_V   ...C...T.C .......CCT .--.....G. ..... ..... ..... G.......-- ---.T..... ..... .....G
[ 800]

#Subtype_I   ATTGAAGGCT TTCGTGTTCG ATTTGATTGG TTTG-TTCAT GGAAGCAAGG TTAAAAGGAA CAGTTGGGGG TATTCATATT CACTAGTTAG AGGTGAAATT
[ 900]
#Subtype_III ..GT.T.AT. ...A..A.GT ..... ....G..... .AG.AT...A ........G. ..... ..... ..A....C.. .....
[ 900]
#Subtype_II  .-GATT.... ...A.AG... ....T-.... ....-..T.. .A.......A ....T...G. ..... ..... ..A...C... .....
[ 900]
#Subtype_V   ..... ..... ..... ....-..... ..... ..... ..... ..... ..... .....
[ 900]

#Subtype_I   CTCGGATTTA TGGAAGATGA ACAAGTGCGA AAGCATTTAC CAAGGATGTT TTCATTAATC AAGAACGAAA GCTAGGGGAT CGAAGAGGAT TAGATACCCT
[1000]
#Subtype_III ..... ..... ..... ..... ..... ..... ..... .T..... ..... .....
[1000]
#Subtype_II  ..AT..... ..... ..T..... ..... ..... ..... ..... ..... .A..... .....
[1000]
#Subtype_V   ..... ..... ...G..... ..... ..... ..... ..... ..... ..... .....
```

```
[1000]

#Subtype_I   CGTAGTCTTA GCTATAAACG ATACCGACTA GGGGTTAGTA GA-GGTCAAA GTGTCTTTAT TAGTACCTTA TGAGAAATCA AAGTCTTTGG GTTCCGGGGG
[1100]
#Subtype_III .......... A......... .......... .A.......G A.-.....TT .......G.C .....T.... .......... .......... ..........
[1100]
#Subtype_II  .......... .......... .......... ..A....... ..T.A..C.. TG..G.C... .......... .......... .......... ..........
[1100]
#Subtype_V   .......... .......... .......... .......... ..-......T ......C... .......... .......... .......... ..........
[1100]

#Subtype_I   GAGTATGGTC GCAAGGCTGA AACTTAAAGG AATTGACGGA AGGGCACCAC CAGGAGTGGA GCCTGCGGCT TAATTTGACT CAACACGGGG AAACTTACCA
[1200]
#Subtype_III .......... .......... .......... .......... .......... .......... .......... .......... .........A ..........
[1200]
#Subtype_II  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
[1200]
#Subtype_V   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
[1200]

#Subtype_I   GGTCCAGACA TAGGAAGGAT TGACAGATTG ATAGCTCTTT CTTGATTCTA TGGGTGGTGG TGCATGGCCG TTCTTAGTTG GTGGAGTGAT TTGTCTGGCT
[1300]
#Subtype_III .......... .......... A........A .-........ .......... .......... .......... .......... .....T.... .....A....
[1300]
#Subtype_II  .......... .......... .......... .......... .......... .......... .......... .......... .......... ........T.
[1300]
#Subtype_V   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
[1300]

#Subtype_I   AATTCCGATA ACGAACGAGA CCTCCGCCT- TTAACTAGT- -------GAC GTGTATTGTG ATATGCGTTG CTTCTTATAG GGACACTATA TGTA-AAATG
[1400]
#Subtype_III .......... .......... .........G C...A.G..- -------TTT C.A..G.A.T C...AG.AAT .C.....G.. .......... CA..-..G.A
[1400]
#Subtype_II  T......... .......... .T.......A ...GT.G.AT GAAATGG..T T.T..GCCCC ..TATTTCGT .AG....G.. .......G.G C..TTG.G.A
[1400]
#Subtype_V   .......... .......... .........- .........- --------.TT .GC....... ...GCT.A.A ......GG.. .......... C...-..G..
[1400]

#Subtype_I   TAGGGAAGCT GGAGGCAATA ACAGGTCTGT GATGCCCTTA GATGTTCTGG GCTGCACGCG CGCGACACTG ATCTATTCAA CGAGTGGCTG GGTCGAGAGA
[1500]
#Subtype_III .......... .......... .......... .......... .....C.... .......... .......... ..TC...... .A........ AA....T...
[1500]
#Subtype_II  C......... ...A...... .......... .......... .......... .......... .......... .A........ .....AT... A.....T...
[1500]
#Subtype_V   .......... .......... .......... .......... .......... .......... .......... .......... .......... T.....T...
```

```
[1500]

#Subtype_I   CTTGGCAAAT CTTGTGAAAG TAGATCGTGA TGGGGATTGA TGCTTGTAAT TGTTCATCAT GAACGAGGAA TTCCTAGTAA ACGCAAGTCA TCAACTTGCA
[1600]
#Subtype_III T......... ...T.....A .GA....... .......... ..TC...... AAA-...... .......... .......... .T........ .........G
[1600]
#Subtype_II  .....G.... ...T.....A ...T...... .......... .......... .T........ .......... .......... .T........ .........G
[1600]
#Subtype_V   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
[1600]

#Subtype_I   TTGATTACGT CCCTGCCCTT TGTACACACC GCCCGTCGCA CCTACCGATT GAATGGTCCG GTGAACACTT TGGATTTGAG ATTGGTTGTC TGTAAAAGGA
[1700]
#Subtype_III .......... .......... .......... .......... .......... .......... A......... .......T.. TAAT..CAGT CT...CG.AT
[1700]
#Subtype_II  .......... .......... .......... .......... .......... .G...A.... .......... .......TTA ...AT..A.. G.CTTGCT.G
[1700]
#Subtype_V   .......... .......... .......... .......... .......... .......... .......... .......T.. GCGAA.GAA. .........G
[1700]

#Subtype_I   TGGTTAAT-C TGGGAGAAGT CGTGTAAATC TTATCATTTA GAGGAAGGTG AAGTCGTAAC AAGGTTTCCG T [1771]
#Subtype_III ..A.G.T.AT ..A....... T......... ...C...... .......... .......... .......... . [1771]
#Subtype_II  .A.A..G.-T GAA....... .......... .......C.. .......... .......... .......... . [1771]
#Subtype_V   GA...CG.-T .......... .......... .......... .......... .......... .......... . [1771]
```

Figure 2 Alignment of the small subunit ribosomal RNA gene of *Blastocystis* from Thai patients. Dots and dashes denote identical residues and deletions, respectively.

Table 1. Coprodiagnosis of parasitic infections in patients at King Chulalongkorn Memorial Hospital, Bangkok from 2001-2005

	2001	2002	2003	2004	2005	Total (%)*
Protozoa						
Blastocystis hominis	234	489	636	681	777	2,817 (25.17)
Giardia intestinalis	257	243	256	253	257	1,266 (11.31)
Entamoeba coli	115	65	136	138	148	602 (5.38)
Endolimax nana	89	92	120	108	163	572 (5.11)
Entamoeba histolytica/dispar	14	37	51	66	80	248 (2.22)
Sacocystis hominis	20	27	32	17	5	101 (0.90)
Trichomonas hominis	36	9	14	9	19	87 (0.78)
Cryptosporidium spp.	21	34	8	9	7	79 (0.71)
Isospora belli	10	17	21	18	11	77 (0.69)
microsporidium	2	11	4	2	0	19 (0.17)
Chilomastix mesnili	1	1	1	1	3	7 (0.06)
Cyclospora cayetanensis	1	2	0	1	1	5 (0.04)
Iodamoeba buetschlii	0	4	0	0	1	5 (0.04)
Helminths						
Strongyloides stercoralis	741	483	453	445	517	2,639 (23.58)
Opisthorchis viverrini	375	199	265	234	146	1,219 (10.89)
hookworm	251	152	173	147	147	870 (7.77)
Taenia saginata	30	44	31	37	63	205 (1.83)
Trichuris trichiura	17	18	36	38	52	161 (1.44)
Ascaris lumbricoides	9	11	7	11	28	66 (0.59)
Enterobius vermicularis	5	32	14	2	1	54 (0.48)
Echinostoma spp.	25	13	4	8	2	52 (0.46)
Capillaria philippinensis	6	7	3	6	4	26 (0.23)
Hymenolepis nana	1	0	2	2	8	13 (0.12)
Fasciolopsis buski	0	2	0	0	1	3 (0.03)
Hymenolepis diminuta	0	1	0	0	0	1 (0.01)
Total positives	2,276	2,014	2,300	2,286	2,460	11,194
Total examined	18,281	15,756	20,510	22,208	32,096	108,851
%Positive	12.45	12.78	11.21	10.29	7.66	10.28

* per cent of all positives for parasites.

Table 2 Distribution of *Blastocystis* subtypes from patients in Thailand

Subtypes*	Patient's profile				Total (%)
	Sex		Symptoms		
	Male	Female	None	Diarrhea	
I	10	15	21	4	25(35.7)
II	-	2	1	1	2 (2.8)
III	11	30	34	7	41(58.6)
IV	-	-	-	-	-
V	2	-	1	1	2 (2.8)
VI	-	-	-	-	-
VII	-	-	-	-	-
Total	23	47	56	13	70

* Subtypes are based on consensus terminology by Stensvold et al Trends Parasitol 2006.

Future Plan:

1. Recruit more samples of *Blastocystis* from human infection for *in vitro* cultivation and sequence analysis.
2. Delineate the possibility of clonal mixture of *Blastocystis* that possesses different small subunit ribosomal RNA sequences by subcloning of the PCR-amplified products into plasmid vectors and sequencing of the recombinant subclones.

Publication list for this work:

Manuscript will be prepared after the completion of this study.

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

Filipinas F. Natividad,[1] Corazon C. Buerano,[1,2] Catherine B. Lago,[1] Cynthia A. Mapua,[1] Blanquita B. de Guzman,[1] Shinji Izumiyama,[3] and Kenji Yagita,[3] Ebonia B. Seraspe,[4] Lorena P. Samentar,[4] Ronald R. Matias,[1] and Takuro Endo[3]

[1] Research and Biotechnology Division, St. Luke's Medical Center, E. Rodriguez Sr. Ave., Quezon City 1102, Philippines, [2] Institute of Biology, University of the Philippines, Diliman, Quezon City 1101, Philippines ,[3] Department of Parasitology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan, [4] Division of Biological Sciences, University of the Philippines in the Visayas, Miag-ao, Iloilo 5023, Philippines

Corresponding author: Filipinas F. Natividad, PhD
Address: Research and Biotechnology Division, St. Luke's Medical Center, E. Rodriguez Sr. Ave., Quezon City 1102, Philippines Telefax: (63) 02-7260467, Email: ffnatividad@stluke.com.ph

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 giardiasis 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 *Giardia* 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 *Cryptosporidium* and *Giardia, 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, brightfield 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 *Rsa*I (Toyobo, Japan), while the nested PCR product was digested with *Ssp*I (Invitrogen) and *Vsp*I (Promega). The PCR and RFLP digested products were fractionated on a 2.0% agarose gel and visualized by ethidium bromide staining using a 2UV™ 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 3456 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 3456 stool samples examined, 133 stools (3.85%) 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; 2.00%) and that of *Cryptosporidium* spp. (67; 1.94%). Out of a total of 133 positive stool samples, 83 (4.98%) were from Luzon, 19 (4.87%) from Mindanao, and 31 (2.22%) 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 ($p=0.930$) in the distribution of positive samples between Mindanao and Luzon. The overall positive rate in the Philippines for *Giardia* and *Cryptosporidium* among pediatric patients was 4.81% and in adults it was 2.09%. Among pediatric patients, the prevalence of *G. lamblia* (1.99%) was significantly lower ($p=0.049$) than that of *Cryptosporidium* spp. (2.92%). On the other hand, the prevalence rates of *Cryptosporidium* spp. (0.24%) and *G. lamblia* (1.93%) 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. (3.12%) was significantly higher than *G. lamblia* (1.92%). However, in Visayas, the prevalence of *G. lamblia* (1.64%) was significantly higher than *Cryptosporidium* spp. (0.64%). There was no sufficient evidence to conclude that the prevalence of *Cryptosporidium* spp. (1.54%) in Mindanao was statistically different from that of *G. lamblia* (3.59%). 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 *Crytosporidium* 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 coinfection 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, 3,456 stool samples came from all major islands in the Philippines. There was no significant difference between the overall prevalence of *G. lamblia* (2.0%) and that of *Cryptosporidium* spp. (1.9%) 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 *Giardia*: 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, *Giardia 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 *Giardia* 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 *Giardia* (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 *Giardia* to decrease with age. Jarmey-Swan *et al.* (30) studying 2,800 children under 5 years in South Africa, indicated that *Cryptosporidium* was most prevalent (39.3%) in the <1 year age group while *Giardia* 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 *Giardia.*

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 *Cryptosporidium* 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 *Giardia* (30).

Our study shows that seasonality in occurrence of *Giardia* 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 *Giardia* 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.

SUMMARY

The prevalence of *Giardia* and *Cryptosporidium* in 3, 456 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.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Epifania S. Simbul (National Children's Hospital), Dr. Carlos M. Oliveros (San Lazaro Hospital) and the Enteric Protozoa Research Network in the Philippines for the collection of stool samples. This study was supported by a project grant of C. C. Buerano from St. Luke's Medical Center Research and Biotechnology Division (05-024) and by a grant to F. Natividad from the Japan Health Sciences Foundation (04-016). This study received support from Ministry of Labor and Welfare of Japan through The National Institute of Infectious Diseases (Tokyo).

REFERENCES

1. The Philippines in Figures 2006. National Statistics Office. Manila, Philippines.
2. Bryan, R. T., Pinner, R. W. and Berkelman, R. L. (1994): Emerging infectious diseases in the United States. Ann. N. Y. Acad. Sci., 740, 346-361.
3. Marshall, M. M., Naumovitz, D., Ortega, Y. and Sterling, C. R. (1997): Waterborne protozoan pathogens. Clin. Microbiol. Rev., 10, 67-85.
4. Cross, J. H., Banzon, T., Wheeling, C. H., Cometa, H., Lien, J. C., Clarke, R., Petersen, H., Sevilla, J. and Basaca-Sevilla, V. (1977): Biomedical survey in North Samar Province, Philippine Islands. Southeast Asian J. Trop. Med. Public Health, 8, 464-475.
5. Carney, W., Banzon, T., de Veyra, V., Papasin, M. and Cross, J. (1981): Intestinal parasites of man in Oriental Mindoro, Philippines, with emphasis on schistosomiasis. Southeast Asian J. Trop. Med. Public Health, 12, 12-18.
6. Adkins, H. J., Escamilla, J., Santiago, L. T., Ranoa, C., Echeverria, P. and Cross, J. H. (1987): Two-year survey of etiologic agents of diarrheal disease at San Lazaro Hospital, Manila, Republic of the Philippines. J. Clin. Microbiol., 25, 1143-1147.
7. Auer C. (1990): Health status of children living in a squatter area of Manila, Philippines, with particular emphasis on intestinal parasitoses. Southeast Asian J. Trop. Med. Public Health, 21, 289-300.
8. Paje-Villar, E., Co, B., Carandang, E., Raymundo, A., Lagamayo, E., Hart, C.A., Lavadia, E. and Rigor, E. M. (1993): Diarrheas among Filipino infants and children: clinical and laboratory correlations. Philipp. J. Pediatr., 42, 1-22.
9. Lee, K.-J., Ahn, Y.-K. and Yong, T.-S. (2000): A small-scale survey of intestinal parasite infections among children and adolescents in Legaspi city, the Philippines. Korean J. Parasitol., 38, 183-185.
10. Carney, W. P., Banzon, T., De Veyra, V., Dana, E. and Cross, J. H. (1980): Intestinal parasites of man in Northern Bohol, Philippines, with emphasis on schistosomiasis. Southeast Asian J. Trop. Med. Public Health, 11, 473-479.
11. Salas, S. R. M. S. (1997): Prevalence of giardiasis among patients at the University of San Agustin Clinical Laboratory. Agustinian, 1, 89-100.
12. Carney, W. P., de Veyra, V. U., Cala, E. M. and Cross, J. H. (1981): Intestinal parasites of man in Bukidnon, Philippines, with emphasis on schistosomiasis. Southeast Asian J. Trop. Med. Public Health, 12, 24-29.
13. Bustos, M. D., Salazar, N., Espino, F. E. C., Montalban, C., Sabordo N. and Laurente, M. (1991): Ornidazole in the treatment of giardiasis in an institution for the mentally retarded. Phil. J. Microbiol. Infect. Dis., 20, 13-16.

14. Baldo, E. T., Belizario, V. Y., De Leon, W. U., Kong, H. H. and Chung, D.I. (2004): Infection status of intestinal parasites in children living in residential institutions in Metro Manila, the Philippines. Korean J. Parasitol., 42, 67-70.
15. Carlos, C. C., Saniel, M. C., Lucero, G. M. and Evangelista, T. A. (1992): Enteropathogens among measles patients with diarrhea in urban Filipino children. Philipp. J. Microbiol. Infect. Dis., 21, 53-60.
16. Cross, J. H., Alcantara, A., Alquiza, L., Zaraspe, G. and Ranoa, C. (1985): Cryptosporidiosis in Philippine children, Southeast Asian J. Trop. Med. Public. Health., 16, 257-260.
17. Laxer, M. A., Alcantara, A. K., Javato-Laxer, M., Menorca, D. M, Fernando, M. T. and Ranoa, C. P. (1990): Immune response to cryptosporidiosis in Philippine children. Am. J. Trop. Med. Hyg., 42, 131-139.
18. Capeding, M. R. and Saniel, M. C. (1990): Cryptosporidiosis in Filipino children: association with acute diarrhea. Philipp. J. Microbiol. Infect. Dis., 19, 49-50.
19. Jueco, N. L., Belizario, V. Y. Jr., de Leon, W. U., Bravo, L. C. and Gregorio, G. V. (1991): Cryptosporidiosis among selected patients in the Philippine General Hospital. Acta Medica Philippina, 27, 244-247.
20. Menorca, D. M., Laxer, M. A., Alcantara, A. K., Javato-Laxer, M., Fernando, M. T. and Gonzales, V. (1994): Statistical analysis of clinical immunological and nutritional factors in pediatric cryptosporidiosis in the Philippines. Southeast Asian J. Trop. Med. Public Health, 25, 300-304.
21. Paje-Villar, E., Co, B. G., Carandang, E. H., Raymundo A., Lagamayo E., Lavadia E., Rigor E. and Hart, C. A. (1994): Non-bacterial diarrhoea in children in the Philippines. Ann. Trop. Med. Parasitol., 88, 53-58.
22. Rivera, W. L., Yason, J. A. D. L. and Rivera, P. T. (2005): Serological detection of cryptosporidiosis among Filipino cancer patients. Parasitol. Res., 98, 75-76.
23. Xiao, L., Fayer, R., Ryan, U. and Upton, S.J. (2004): *Cryptosporidium* taxonomy: recent advances and implications for public health. Clin Microbiol Rev. 17(1):72-97.
24. Yagita, K., Izumiyama, S., Tachibana, H., Masuda, G., Iseki, M., Furuya, K., Kameoka, Y., Kuroki, T., Itagaki, T. and Endo, T. Molecular characterization of Cryptosporidium isolates obtained from human and bovine infections in Japan. Parasitol Res. 2001 Nov;87(11):950-5.
25. Xiao, L., Escalante, L., Yang, C., Sulaiman, I., Escalante, A.A., Montali, R.J., Fayer, R. and Lal, A.A. (1999): Phylogenetic analysis of *Cryptosporidium* parasites based on the small-subunit rRNA gene locus. Appl Environ Microbiol. 65(4):1578-83.
26. Cross, J. and Basaca-Sevilla, V. (1984): Biomedical surveys in the Philippines. Manila US Naval Medical Research Unit No. 2 SP-47, 117.