รายงานวิจัยฉบับสมบูรณ์

โครงการ การศึกษาสายพันธุ์ของเชื้อ Giardia duodenalis ในประเทศไทย

โดย

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Executive Summary

1. Project description

Objectives and rationale

Giadiasis is one of the most frequently diagnosed intestinal protozoa infection caused by *Giardia duodenalis*. Prevalence of *G. duodenalis* is found in all age groups but children are at greatest risk for contracting clinical giardiasis especially those attending day care centers, living in poor hygienic conditions or in community settings. In young children, *G. duodenalis* causes diarrhoea and impairs gain of body weight which interferes with growth and development. In Thailand, prevalence of giardiasis has been studied in different groups of population which was 21 % in school children (1984). Prevalence surveys in orphans in 1990 and 2001 were 20 % and 12 %, respectively. In addition, study of giadiasis in military personnel was 1-3 % during 1998-2001. Giardiasis is treatable but our study found the reinfection was as high as the prevalence reported before treatment. Thus, treatment alone is not the only implement to control the infection. Understanding the epidemiology of *G. duodenalis* can give a significant impact on developing methods of control and prevention, especially in high risk groups.

G. duodenalis is found not only in humans but also in other mammals including pets, livestocks; i.e. dogs, cats, cattle, pigs, sheep, goat and horses which the organism has morphologically identical. Diagnosis of *Giardia* is based on light microscopic method but it is of limited epidemiological value. Using molecular tools such as the Polymerase Chain Reaction (PCR) with specific genes, genetic diversity within *G. duodenalis* have been demonstrated. The ability to accurately genotype *Giardia* not only determines sources of infection in outbreak situations but also provides direct evidence of zoonotic transmission. Several specific genes have been studied but reliable markers are needed to be investigated which can detect variation within the genotypes of *Giardia* known to infect humans and animals. In addition, the method should be proven to be reproducible and ideal for routine genotyping. We propose the investigation of the difference within Thai strains of *G. duodenalis* by using the PCR and sequencing of the multi-loci genes encoding for SSU-rDNA, elongation factor 1-alfa (*ef1-*α*),* triose phosphate isomerase (*tpi*), and PCR-RFLP (Random-Fragment-Length-Polymorhism) of the glutamate dehydrogenase (*gth*) gene.

Understanding the genetic variation of *G. duodenalis* among different populations will be useful in exploring the source of infection and transmission.

2. Aims of the study

1 To investigate the existence of genetic variation within Thai strains of *G. duodenalis* isolated from different groups of Thai population and from potentially zoonotic animals using the PCR. A genotypic study using multi- gene loci i.e. SSU-rDNA, elongation factor 1-alfa (*ef1-*α), triose phosphate isomerase (*tpi*), and glutamate dehydrogenase for genotypic characterization will be performed in this study. Correlation of the genotypes with each specific gene will be studied using phylogenetic analysis.

2. Each genotype of *Girdia* will be compared to each individual data of health outcomes and risk factors, which could provide the epidemiological information including sources of transmission and association of genotypes and clinical outcomes of the organism.

สรุปผลการดำเนินงาน

1. Evaluations of sensitivities of specific genes for genotypic characterization of *Giardia duodenalis*

เนื่องจากการศึกษานี้จะใช้ specific genes 5 loci ที่จะนำมาศึกษา genotypes ของ *G. duodenalis* จากอุจจาระ คือ small subunit- rDNA (SSU-rDNA), glutamate dehydrogenase (*gdh*), elongation factor-1a (elf-1 α), triose phosphate isomerase (tpi), β -giardin (β -giardin gene) จึงต้องเลือกใช้วิธีการสกัด DNA จากเชื้ออุจจาระที่เหมาะสมที่สุด เนื่องจากมี inhibitors ในอุจจาระหลายชนิดที่ทำให้การสกัด DNA ได้ผลไม่ดีนัก และ เลือก gene ที่มีความไว (sensitivity) ดีที่สุดจากทั้งหมด 5 loci ก่อนเป็นลำดับแรกเพื่อใช้ในการ detect เชื้อจากอุจจาระ

<u>เตรียม trophozoite และ cyst ที่ทราบจำนวนโดยนำไปนับ หรือวัด OD</u>

1. เตรียม trophozoite ของ *G. duodenalis* จาก axenic culture (เตรียม trophozoite จาก axenic culture โดยวิธีปั่นล้างด้วย PBS หลายรอบ นำมา freeze-thawed เพื่อให้ trophozoite แตก และนำไปหาค่าความเข้มข้นของ DNA โดยการวัดค่า optical density จากนั้นทำ dilution ให้ได้ปริมาณ DNA 1pg/ ml,10 pg/ ml, 100pg/ ml, 100ng/ ml,10 ng/ ml และ 1 ng/ ml ตามลำดับ

2. เตรียมcyst ของ *G. duodenalis* จาก stool specimen (เตรียม cyst ปริมาณมากจากอุจจาระโดยวิธี Floatation technique ด้วย Sodium nitrate และล้างด้วย PBS หลายๆรอบก่อนนำมานับจำนวน cyst โดยใช้ hemocytometer และคำนวณให้มีปริมาณ 84,210 cyst/ ml จากนั้นทำ dilution ให้ได้ปริมาณ 3368, 674, 337,168, 84 cyst/ ml ตามลำดับ

<u>PCR protocols for 5 specific loci</u>

ใช้ *G. duodenalis* จากที่เตรียมได้นำมาทดสอบ PCR methods โดยใช้ target genes 5 loci ดังนี้ small subunit- rDNA (SSU-rDNA), glutamate dehydrogenase (*gdh*), elongation factor-1a (elf-1 α), triose phosphate isomerase (*tpi*), β -giardin (β -giardin gene) โดยใช้ specific primers (ดูรูป1-5)

Target gene	Primers	Method	References
SSU-rDNA	RH11, 5'-CAT CCG GTC GAT CTC TGC C -3' RH4, 5'-AGT CGA ACC CTG ATT CTC CGC CCA GG -3' GiarF, 5'-GAC GCT CTC CCC AAG GAC -3' GiarR, 5'-CTG CGT CAC GCT GCTCG -3'	the nested PCR and sequencing	Hopkins et al., 1997, Read et al., 2002

glutamate dehydrogenase (*gdh*)	GDHeF: 5'-TCA ACG AYA AYC GYG GYT TCC GT-3' GDHiF: 5'-CAG TAC AAC ACY GCT CTC GG-3' reverse primer ,GDHiR: 5'-GTT RTC CTT GCA CAT CTC C-3'	The nested PCR and sequencing together with PCR-RFLP	Read *et al*., 2004
elongation factor 1- α (*ef1-α*)	EF1AR, 5'-AGCTCYTCGTGRTGCATYTC- 3' GLONGF, 5'-GCTCSTTCAAGTACGCGTGG-3' RTeF1-aF, 5'-GCC GAG GAG TTC GAC TAC ATC -3' RTeF1-aR, 5'-GAC GCC SGA GAT CTT GTA GAC -3'	PCR with sequencing	Monis et al., 1999 Traub et al., 2004
triose phosphate isomerase (*tpi*)	AL3543 , 5′- AAATIATGCCTGCTCGTCG-3′ AL3546, 5′- CAAACCTTITCCGCAAACC-3′ AL3544, 5′- CCCTTCATCGGIGGTAACTT-3′ AL3545, 5′- GTGGCCACCACICCCGTGCC-3′	Nested PCR with sequencing	Sulaiman et al., 2003
β-Giardin	G7 5′- AAGCCCGACGACCTCACCCGCAGTGC-3′ G759 5′- GAGGCCGCCCTGGATCTTCGAGACGAC-3′ Forward primer, 5'-GAACGAGATCGAGGTCCG-3' Reverse primer, 5'-CTCGACGAGCTTCGTGTT-3'	Nested PCR with sequencing	Caccio et al., 2002 Lalle et al., 2004

ตารางที่ 1 : ศึกษา Target gene ที่มีความไว (sensitivity) ที่ดีที่สุดที่สามารถ detect เชื้อในอุจจาระ (ทั้ง trophozoite และ cyst (ดูรูป1-5)

Target gene	1 pg of trophozoite	10 pg of trophozoite	100 pg of trophozoite	1 ng of trophozoite	10 ng of trophozoite	100 ng of trophozoite
SSU-rDNA	-	+	+	+	+	+
glutamate dehydrogenase (*gdh*)	-	-	-	+	+	+
triose	-	-	-	+	+	+

phosphate isomerase (*tpi*)						
elongation factor 1-alfa (*ef1*- α)	-	-	-	-	+	+
β -Giardin	-	-	+	+	+	+

Pg = picrogram of DNA, ng = nanogram of DNA

Done in dublicate

ตารางที่ 2 ประเมินวิธีการสกัด DNA 2 วิธี (QAIGEN stool miniKit กับ FTA assay)

เลือกวิธี DNA extraction ที่เหมาะสมที่สุดเพื่อใช้ extract DNA ของเชื้อ *G. duodenalis* จากอุจจาระก่อนทำ PCR ซึ่งได้ทำการเปรียบเทียบ sensitivity ของ 2 วิธี คือ QAIGEN stool miniKit (QIAGEN, Germany) กับ FTA assay (Whatman, England) โดยดูผล PCR ของ SSU-rDNA

DNA extraction method	168 cysts/ml	337 cysts/ml	674 cysts/ml	3368 cysts/ml
QIAMP stool miniKit	-	-	-	+
FTA method	+	+	+	+

สรุป: ระยะ cyst (ซึ่งโดยทั่วไปเป็นระยะที่ตรวจพบในอุจจาระมากกว่าระยะ trophozoite) มีผนังหนาแตกค่อนข้างยาก มักมีปัญหาในการสกัดแยก DNA ประกอบกับอุจจาระมีไขมัน และ inhibitor อื่นๆ การใช้ FTA assay ในการสกัดแยก DNAจาก ระยะ cyst ของ *G duodenalis.* พบว่าให้ผลดีกว่า QAIGEN stool miniKit เมื่อศึกษาเปรียบเทียบกัน ด้วยการทำ PCR โดยใช้ gene SSU-rDNAพบว่า FTA assay สามารถ detect ระยะ cyst ได้จำนวนน้อยที่สุดที่ 168 cyst/ ml ส่วน QAIGEN stool miniKit assay สามารถ detect ระยะ cyst ได้จำนวนน้อยที่สุดที่ 3368 cyst/ ml ดังนั้นจึงได้เลือก FTA assayในการหา sensitivities (ความไว) ของ gene ทั้ง 5 loci

<u>เลือก specific genes ที่มี sensitivity ดีที่สุดในการตรวจหาร cyst จากอุจจาระ (จากทั้งหมด 5 loci)</u>

หมายถึง specific gene ที่ สามารถ detect ปริมาณ cyst จำนวนน้อยที่สุดของ *G. duodenalis* ที่ตรวจพบจากอุจจาระได้ ผลจากการศึกษาเพื่อเลือก gene ที่มี sensitivity ดีที่สุด เพื่อนำไปใช้ในการ detect เชื้อในอุจจาระต่อไป (screening)

ตารางที่ 3. FTA assay สกัดแยก DNA จาก cyst ซึ่งตรวจพบ sensitivities ของ Target genes ดังนี้

Target genes*	168 cysts/ml	337 cysts/ml	674 cysts/ml	3368 cysts/ml
SSU-rDNA	+	+	+	+
glutamate dehydrogenase (*gdh*)	-	+	+	+
triose phosphate isomerase (*tpi*)	-	-	+	+
elongation factor 1-alfa (*ef1*- α)	-	-	-	-
β -Giardin	-	-	-	-

*Done in duplicate

<u>ผลการศึกษา</u>: SSU-rDNAให้ผล sensitivity ดีที่สุดในการตรวจหาเชื้อระยะ cyst จากอุจจาระ โดยผลจากการทำ PCR สามารถตรวจหาปริมาณ cyst ได้ 168 cysts/ml โดย target geneที่มี sensitivity รองลงมาคือ glutamate dehydrogenase (*gdh*)สามารถตรวจหาปริมาณ cyst ได้ 337 cysts/ml เมื่อเทียบกับ triose phosphate isomerase (*tpi*), elongation factor 1-alfa (*ef1*- α) และ β -Giardin โดยเฉพาะ elongation factor 1-alfa (*ef1*- α) และ β -Giardin มี sensitivity ต่ำสุดในการตรวจหา cyst ซึ่งเป็นระยะที่พบเป็นส่วนใหญ่

II. คณะผู้วิจัยได้เปรียบเทียบ sensitivity และ specificity ของวิธี PCR โดยใช้ SSU-rDNA ซึ่งเป็น target gene ที่ดีที่สุดในการตรวจเชื้อ *G. duodenalis* โดยเปรียบเทียบกับวิธี indirect fluorescence assay (IFA) โดยใช้อุจจาระที่ตรวจพบเชื้อ *G. duodenalis* ด้วยวิธี floatation concentration และไม่พบเชื้อ รวมจำนวน 70 ตัวอย่าง และได้ใช้วิธี double blind method ซึ่งผู้ทำ PCR (specific primer ต่อ SSU-rDNA gene) และวิธี indirect fluorescence assay (IFA) เป็นคนละคนกัน และทั้งสองคนไม่ทราบผลการอุจจาระที่ตรวจโดยวิธี floatation concentration มาก่อนเพื่อมิให้เกิดการ bias ในการตรวจ ผลการศึกษามีดังนี้

ตารางที่ 4 เปรียบเทียบวิธี PCR and IFA ในการตรวจหา *G. duodenalis* ในตัวอย่างอุจจาระ 70 ตัวอย่าง

Method	Result	Number and (%)		
		Positive by concentration	Negative by concentration	Investigated
PCR*	Positive	36 (97.29)	0 (0)	36 (51.43)
	Negative	1 (2.7)	33 (100)	34 (48.57)
IFA*	Positive	34 (91.89)	0 (0)	34 (48.57)
	Negative	3 (8.11)	33 (100)	36 (51.43)
PCR or IFA	Any	37 (52.8)	33 (47.1)	70 (100)

*Done in duplicate

เปรียบเทียบ Sensitivity และ Specificity ของ PCR และ IFA

เมื่อใช้ floatation concentration เป็น gold standard ผล Sensitivity ของวิธี PCR คือ 97.29% (95%CI, 87.39-99.86) และ IFA 91.89% (95%CI, 79.50-97.89) ตามลำดับ เมื่อใช้เชื้อที่ตรวจพบและไม่พบในอุจจาระด้วยวิธี floatation concentration โดยทั้ง 2 วิธีให้ผล specificity 100% (95%CI, 91-100) ความแตกต่างของ Sensitivity เมื่อเปรียบเทียบทั้ง 2 วิธี เป็น 5.41 % (95%CI, 0.39-99.86) ซึ่งไม่มีนัยสำคัญทางสถิติ ($P = 0.61$)

***ผลการศึกษาได้ตีพิมพ์ใน Journal of Clinical Microbiology (ภาคผนวก)**

Nantavisai K, Mungthin M, Tan-ariya P, Rangsin R, Naaglor T, Leelayoova S. Evaluation of the sensitivities of DNA extraction and PCR methods for detection of *Giardia duodenalis* in stool specimens. Journal of Clinical Microbiology 2007; 45: 581-583."

2. การสำรวจพยาธิในลำไส้ในทหาร ที่ จ นครราชสีมา ได้ดำเนินการตรวจอุจจาระจากกำลังพลทั้งหมดจำนวน317 นาย โดยวิธี wet preparation, floatation/sedimentation technique, in vitro culture for *Blastocystis hominis*, modified acid fast stain for *Cryptosporidium*, gram-chromatrope for Microsporidia และข้อมูลจากแบบสอบถามได้เก็บโดยใช้โปรแกรม SPSS

ตาราง 5. แสดงความชุกของโรคพยาธิลำไส้ทหาร ที่ จ นครราชสีมา ที่ทำการศึกษา 317 ราย ตรวจพบพยาธิลำไส้ 72 ราย (22.4%)

Intestinal parasite	Positive	Percent
Military Personnel (N=317)		
Blastocystis sp.	46	14.5
Strongyloides stercoralis	8	2.5
Giardia duodenalis	4	1.3
Hookworm	3	1.0
Opisthorchis viverrini	3	1.0
Taenia spp.	1	0.3
Entamoeba coli	7	2.2
Endolimax nana	2	0.6
Total	71	22.4
Military dogs (N=189)		
Blastocystis sp.	5	2.6
Strongyloides stercoralis	1	0.5
Entamoeba coli	1	0.5
Total	7	3.7

*เป็นเชื้อที่ก่อให้เกิดพยาธิสภาพในลำไส้ (pathogenic intestinal parasites) ทุกรายได้รับยาฆ่าพยาธิตามมาตรฐาน

จากกำลังพลที่ได้รับการตรวจอุจจาระทั้งหมด 317 ราย พบความชุกการติดเชื้อพยาธิในลำไส้ทั้งหมด 72 ราย คิดเป็น 22.4 % พบเชื้อ *Blastocystis hominis* สูงสุดจำนวน 46 รายคิด เป็น 14.5 % และพบเชื้ออื่นๆ ตามลำดับดังนี้ *Strongyloides stercoralis*, *Entamoeba coli, Giardia duodenalis*, Hookworm, *Opisthorchis viverrini, Taenia* sp. *, Endolimax nana* (แสดงในตารางที่ 5) คณะผู้วิจัยได้ให้การรักษาแก่กำลังพลที่จำเป็นต้องได้รับฆ่าพยาธิทุกนาย

กำลังพลที่ไม่ได้รับยาฆ่าพยาธิ เป็นกลุ่มที่พบเชื้อ *Blastocystis hominis, Entamoeba coli และ Endolimax nana* เนื่องจากเชื้อเหล่านี้ไม่ทำให้เกิดอาการใดๆ แต่บ่งบอกถึงสุขอนามัยของกำลังพลที่ไม่ดีพอ เช่น การดื่มน้ำที่ไม่สะอาด เนื่องจากเชื้อเหล่านี้สามารถปนเปื้อนในอาหารหรือน้ำดื่มได้

คณะผู้วิจัยได้แจ้งผลการตรวจอุจจาระแก่กำลังพลทั้งหมด รวมทั้งแจกเอกสารให้ความรู้ ด้านสุขศึกษา การดูแลตนเอง การดื่มน้ำและอาหารที่สะอาด การป้องกันการติดเชื้อพยาธิทุกชนิดแก่กำลังพลที่เข้าร่วมโครงการเพื่อลดการติดเชื้อให้ได้มากที่สุด

สุนัขทหารได้รับการตรวจอุจจาระทั้งสิ้น 189 ตัว พบความชุกการติดเชื้อพยาธิในลำไส้ทั้งหมด 7 ตัวคิดเป็น 3.7% โดยพบ *Blastocystis hominis* (5 , 2.6%) และ *Strongyloides stercoralis* (1, 0.5%), *Entamoeba coli* (1, 0.5%) ในระหว่างการสำรวจได้แจ้งผลการตรวจอุจจาระให้สัตว์แพทย์ที่ดูแลรับทราบเพื่อให้ยาฆ่าพยาธิกับสุนัขต่อไป ผลจากการศึกษาครั้งนี้แสดงความชุกการติดเชื้อพยาธิในลำไส้ในสุนัขน้อยมาก แสดงให้เห็นถึงการเอาใจใส่และดูแลสุนัขทหารเป็นอย่างดี รวมทั้งการให้ยาถ่ายพยาธิตามกำหนดเวลาอย่างต่อเนื่องโดยสัตว์แพทย์ที่ดูแล

เชื้อ Genotype ของ *Giardia duodenalis* จากตัวอย่างที่ตรวจพบในทหารขณะนี้มี 1 กลุ่ม คือ **BIV** เป็นกลุ่มที่พบและมีรายงานในคน

***ผลการศึกษาได้ตีพิมพ์ใน Journal Medical Association of Thailand (ภาคผนวก)**

Saovanee Leelayoova, Suradej Siripattanapipong, Tawee Naaglor, Paanjit Taamasri, Mathirut Mungthin. Prevalence of Intestinal Parasitic Infections in Military Personnel and Military Dogs, Thailand. Journal Medical Association of Thailand (*in press* 2009)

3. การสำรวจพยาธิในลำไส้ที่ ดำเนินการที่โรงเรียนบ้านนายาว ต. ท่ากระดาน อ. สนามชัยเขต จ. ฉะเชิงเทรา

ตารางที่ 6 แสดงความชุกของโรคพยาธิลำไส้ในเด็กนักเรียนระดับประถมศึกษาโรงเรียนบ้านนายาว ท่ากระดาน จังหวัดฉะเชิงเทรา ได้ดำเนินการตรวจอุจจาระจำนวนทั้งสิ้น 531ราย ตรวจพบพยาธิลำไส้ 134 ราย (25.2%)

เชื้อที่ตรวจพบในอุจจาระ	จำนวน	ร้อยละ
Blastocystis hominis	43	8.1
**Giardia duodenaliss*	33	6.2
**Opisthorchis. viverrini*	21	3.9
**Trichuris trichiura*	8	1.5
*Hookworm	7	1.3
**Enterobius vermicularis*	3	0.6
**Strongyloides stercoralis*	2	0.4
Other parasites	17	3.2

*เป็นเชื้อที่ก่อให้เกิดพยาธิสภาพในลำไส้ (pathogenic intestinal parasites) ทุกรายได้รับยาฆ่าพยาธิตามมาตรฐาน

ผลการศึกษา *G. duodenalis* ที่ตรวจพบเฉพาะในนักเรียนที่โรงเรียนบ้านนายาว จ ฉะเชิงเทรา นำมาวิเคราะห์ข้อมูลเพื่อหาปัจจัยเสี่ยงของการติดเชื้อ (risk factors) ผลของ Assemblage ที่ศึกษาโดยใช้ glutamate dehydrogenase (*gdh*) พบว่า *G. duodenalis* ที่ตรวจพบในโรงเรียนแห่งนี้เป็น Assemblage A subgenotype II และ Assemblage B subgenotype IV ซึ่งการติดต่อของ Assemblage A subgenotype II ในนักเรียนอาจเกิดขึ้นจากการดื่มน้ำ ปัจจัยเสี่ยงของการติดเชื้อพบหลายชนิดร่วมกัน (multiple mode of transmission) เมื่อวิเคราะห์ข้อมูลโดยทำ multiple regression แล้ว ปัจจัยเสี่ยงที่สำคัญคือ เด็กอายุ 5-9 ปี ในครอบครัวมีสมาชิกที่เป็นเด็กเท่ากับหรือมากกว่า 3 คนขึ้นไป บิดามารดาไม่ได้เรียนหนังสือ ดื่มน้ำขวดที่ผลิตขึ้นเองในชุมชน มีการสัมผัสและใกล้ชิดสุนัข

***ผลการศึกษาได้ตีพิมพ์ใน American Journal of Tropical Medicine and Hygiene (ภาคผนวก)**

Supawat Ratanapo, Mathirut Mungthin, Sutapong Soontrapa, Chakri Faithed, Suradej Siripattanapipong, Ram Rangsin, Tawee Naaglor, Phunlerd Piyaraj, Paanjit Taamasri, **Saovanee Leelayoova.** Multiple modes of Transmission of giardiasis in primary school children of rural community, Thailand. American Journal of Tropical Medicine and Hygiene 2008; 78:611-615.

4. คณะผู้วิจัยได้เก็บตัวอย่างน้ำดื่มในโรงเรียนทุกจุด เพื่อตรวจหาแหล่งติดเชื้อของโปรโตซัวในลำไส้ในโรงเรียน นำน้ำดื่มมากรอง เพื่อสกัด DNA และทำ PCR เพื่อหาการติดเชื้อทั้ง *G. duodenalis*, *Blastocystis hominis* จากการศึกษาตรวจไม่พบเชื้อ *G. duodenalis* ด้วย PCR แต่พบ *Blastocystis hominis* ซึ่งตรวจ genotype แล้วพบตรงกับเชื้อ *Blastocystis hominis* ที่พบในอุจจาระของเด็กนักเรียน ข้อมูลนี้ถึงแม้จะไม่ใช่ข้อมูลโดยตรงว่ามี *G. duodenalis* ปนเปื้อนมากับน้ำ แต่เมื่อวิเคราะห์ข้อมูลทางสถิติพบว่าเด็กนักเรียนที่ติดเชื้อ *Blastocystis hominis* มีโอกาสติดเชื้อ *G. duodenalis* ร่วมด้วย ($p<0.001$) ข้อมูลวิจัยนี้ยืนยันการติดเชื้อโปรโตซัวที่ปนเปื้อนกับน้ำดื่มซึ่งมีประโยชน์อย่างยิ่งในการให้คำแนะนำป้องกันการติดเชื้อโปรโตซัวในลำไส้ให้แก่เด็กนักเรียนในโรงเรียน และชุมชน

ผลการศึกษาได้ตีพิมพ์ใน American Journal of Tropical Medicine and Hygiene (ภาคผนวก)

Leelayoova S, Siripattanapipong S, Thathaisong U, Naaglor T, Taamasri P, Piyaraj P, Mungthin M. Drinking Water, a Possible Source of *Blastocystis* subtype 1 Infection in Schoolchildren of a Rural Community, Central Thailand. American Journal of Tropical Medicine and Hygiene 2008; 79: 401-

5. ถึงแม้ว่าผลการศึกษาพบว่าการdetect เชื้อ *G. duodenalis* ด้วย SSU-rDNAให้ผล sensitivity ดีที่สุดในการตรวจหาเชื้อระยะ cyst จากอุจจาระ และ target geneที่มี sensitivity รองลงมาคือ glutamate dehydrogenase (*gdh*) คณะผู้วิจัยเลือกใช้ glutamate dehydrogenase (*gdh*) ในการศึกษา genotypes ของ *Giardia duodenalis* ในประชากรที่ตรวจพบเนื่องจากสามารถทำ PCR-RFLP ได้ทันทีและสามารถแยกสายพันธุ์ได้ทุก assemblage จากการใช้ Enzyme *Nla*IV และ *Rsa*I ตัด PCR product ซึ่งขณะนี้ได้ทำ PCR และ PCR-RFLP รวมทั้ง sequence PCR products เพื่อยืนยันผลของ PCR-RFLP

สำหรับตัวอย่างเชื้อที่เก็บจากอุจจาระและไม่สามารถทำ PCR amplification ได้ทั้งหมด เนื่องจาก sensitivity ของ glutamate dehydrogenase (*gdh*) gene ที่ใช้ไม่สูงมากพอ ดังนั้นคณะผู้วิจัยได้ทดลองปรับเพิ่ม primers โดย modify primary primers ใหม่เพิ่มเข้าไป คือ GDH1a /GD1s ใส่เพิ่มใน first-round PCR และทำ second-round PCR ตามโดยใช้ primer set GdHeF/GDHiR ที่รายงานโดย Read et al 2004 ซึ่งสามารถ amplify ได้ PCR product สุดท้ายได้ชัดเจน และสามารถใช้ Enzyme *Nla*IV และ *Rsa*I ในการทำ PCR-RFLP เพื่อแยกสายพันธุ์ได้เหมือนเดิม

ในการทดลองได้ใช้ตัวอย่างเชื้อ *Giardia duodenalis* ทั้งสิ้น93 ตัวอย่าง ศึกษา primers ที่ออกแบบคู่ใหม่เพิ่มไปเป็น GDH1 /GDH1a /GD1s เปรียบเทียบกับ primer เดิมGDH1/GDH4 โดยทั้ง 2 วิธีจะทำ secondary PCR ซึ่งใช้ primers GdHeF/GDHiR (Read et al 2004)(ดูตารางที่ 7)

ตารางที่ 7. Nested-PCR of Glutamate dehydrogenase *(gdh)* to detect *G. duodenalis* cysts in faecal specimens

PCR	Primers	PCR product (bp)	References
Primary	Forward/ GDH1 (5'ATC TTC GAG AGG ATG CTT GAG3') Forward/ GDH1a (5'ATC TTC GAG AGG GAT GCT TGA G3') Reverse/ GDH5s (5'GGA TAC TTS TCC TTG AAC TC3')		This paper
Secondary	Forward/ GDHeF (5'TAC ACG TYA AYC GYG GYT TCC GT3') Reverse/ GDHiR (5'GTT RTC CTT GCA CAT CTC C3')	432	Read *et al*. 2004
Primary	Forward/ GDH1 (5'ATC TTC GAG AGG ATG CTT GAG3') Reverse/ GDH4 (5'AGT ACG CGA CGC TGG GAT ACT3')	769	Homan *et al.* 1998
Secondary	Forward/ GDHeF (5'TAC ACG TYA AYC GYG GYT TCC GT3') Reverse/ GDHiR (5'GTT RTC CTT GCA CAT CTC C3')	432	Read *et al*. 2004

ในการทดลองได้ใช้ตัวอย่างเชื้อ *Giardia duodenalis* ทั้งสิ้น93 ตัวอย่าง ศึกษา primers ที่ออกแบบคู่ใหม่เพิ่มไปเป็น GDH1 /GDH1a /GD1s เปรียบเทียบกับ primer เดิมGDH1/GDH4 โดยทั้ง 2 วิธีจะทำ secondary PCR ซึ่งใช้ primers GdHeF/GDHiR (Read et al 2004)(ดูตาราง) ผลการศึกษาสามารถเพิ่ม sensitivity ในการ amplify GDH gene ได้มากขึ้นกว่าเดิม และมีความแตกต่างทางสถิติเมื่อเทียบกับ Primer เดิมที่ใช้อยู่ ขณะนี้ได้ส่งตีพิมพ์แล้ว

ผลการศึกษาอยู่ในระหว่างส่งตีพิมพ์ (ภาคผนวก)

Boontanom P, Siripattanapipong S, Mungthin M, Tan-ariya P, **Leelayoova S** Improved Sensitivity of PCR Amplification of Glutamate Dehydrogenase Gene for the Detection *Giardia duodenalis* in Stool Specimens.

6. สรุปผล assemblage ของ *Giardia duodenalis* ในประชากรกลุ่มอื่นๆ

สรุปรายงานตั้งแต่หน้า 15-48 โดยรวบรวมผลการศึกษาในกลุ่มประชากร 4 กลุ่ม ซึ่งสามารถแยกเรื่องเป็นหัวข้อย่อยๆในการตีพิมพ์ต่อไป เนื่องจากพบ assemblage ของเชื้อและมีปัจจัยเสี่ยงการติดเชื้อแตกต่างกันเช่น ประชากรในกลุ่ม เด็กอ่อนปากเกร็ด จ. นนทบุรี (Cohort study), เด็กชาวเขา อำเภอแม่แจ่ม และ อำเภอฮอด จ. เชียงใหม่ และ เด็กอนุบาล สนามชัยเขต จ. ฉะเชิงเทรา (cross-sectional study) เป็นต้น

XII. Outcome ทั้งหมด

1. Presentation (ภาคผนวก)

1.1 Poster Presentation ครั้งที่ 1 “Evaluation of the sensitivities of DNA extraction and PCR methods for detection of *Giardia duodenalis* in stool specimens” ในการประชุม “นักวิจัยรุ่นใหม่พบเมธีวิจัยอาวุโส สกว.” ที่ โรงแรมรีเจนท์ ชะอำ วันที่ 12-14 ต.ค. 49

1.2 Poster Presentation ครั้งที่ 2 “Multiple modes of Transmission of giardiasis in primary school children of rural community, Thailand” ในการประชุม “นักวิจัยรุ่นใหม่พบเมธีวิจัยอาวุโส สกว.” ที่ โรงแรมแอมบาสซาเดอร์ ซิตี้ จอมเทียน จ. ชลบุรี วันที่ 11-13 ต.ค. 50

1.3 Oral Presentation “Multiple modes of Transmission of giardiasis in primary school children of rural community, Thailand” ในการประชุมวิชาการนานาชาติ “Joint International Tropical Medicine Meeting 2007” ที่โรงแรมอิมพีเรียล ควีนส์ปาร์ก กรุงเทพ วันที่ 29-30 พ.ย.50

1.4 Oral Presentation ในการประชุมวิชาการนานาชาติ “The Third ASEAN Congress of Tropical Medicine and Parasitology (ACTMP3)” ที่โรงแรมวินเซอร์ กรุงเทพ วันที่ 22-23 พ.ค. 51

1.5 Oral Presentation ในการประชุมวิชาการนานาชาติ “Joint International Tropical Medicine Meeting 2008” ที่โรงแรมอิมพีเรียล ควีนส์ปาร์ก กรุงเทพ วันที่ 13-14 ต.ค.51

2. Published paper 4 เรื่อง (ภาคผนวก)

2.1 Nantavisai K, Mungthin M, Tan-ariya P, Rangsin R, Naaglor T, **Leelayoova S.** Evaluation of the sensitivities of DNA extraction and PCR methods for detection of *Giardia duodenalis* in stool specimens. Journal of Clinical Microbiology 2007; 45: 581-583.” (Impact factor = 3.445)

Corresponding author: Leelayoova S.

ผู้นิพนธ์ชื่อแรก : น.ส. ขวัญนันท์ นันทวิศัย (Nantavisai K) นักศึกษาปริญญาโท ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ ม. มหิดล, ขณะนี้กำลังศึกษาต่อระดับปริญญาเอกที่ University of Liverpool, United Kingdom.

2.2 Supawat Ratanapo, Mathirut Mungthin, Sutapong Soontrapa, Chakri Faithed, Suradej Siripattanapipong, Ram Rangsin, Tawee Naaglor, Phunlerd Piyaraj, Paanjit Taamasri, Saovanee Leelayoova. Multiple modes of Transmission of giardiasis in primary school children of rural community, Thailand. American Journal of Tropical Medicine and Hygiene 2008; 78:611-615. (impact factor = 2.546)

Corresponding author: Saovanee Leelayoova

ผู้นิพนธ์ชื่อแรก : นายสุภวัฏ รัตนาโภ ทำวิจัยขณะเป็น นักเรียนแพทย์ทหารชั้นปีที่ 4 วิทยาลัยแพทยศาสตร์พระมงกุฎเกล้า

2.3 Leelayoova S, Siripattanapipong S, Thathaisong U, Naaglor T, Taamasri P, Piyaraj P, Mungthin M. Drinking Water, a Possible Source of *Blastocystis* subtype 1 Infection in Schoolchildren of a Rural Community, Central Thailand. American Journal of Tropical Medicine and Hygiene 2008; 79: 401-6. (Impact factor = 2.546)

Corresponding author: Saovanee Leelayoova

2.4 Saovanee Leelayoova, Suradej Siripattanapipong, Tawee Naaglor, Paanjit Taamasri, Mathirut Mungthin. Prevalence of Intestinal Parasitic Infections in Military Personnel and Military Dogs, Thailand. Journal Medical Association of Thailand (*in press*)

Corresponding author: Saovanee Leelayoova

5. Manuscript (submitted) 1 เรื่อง

Improved Sensitivity of PCR Amplification of Glutamate Dehydrogenase Gene for the Detection *Giardia duodenalis* in Stool Specimens. Boontanom P, Siripattanapipong S, Mungthin M, Tan-ariya P, **Leelayoova S**

Corresponding author: Saovanee Leelayoova

ผู้นิพนธ์ชื่อแรก : ปริมา บุญถนอม (Boontanom P) ขณะนี้กำลังศึกษาระดับปริญญาโทที่ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ ม. มหิดล

พันเอกหญิง เสาวนีย์ ลีละยูวะ
(หัวหน้าโครงการวิจัยผู้รับทุน)
28 กุมภาพันธุ์ 2552

Molecular Epidemiology of *Giardia duoenalis* Infection in Various Groups of Thai Population

INTRODUCTION

Giardia is an intestinal flagellated protozoan parasite that infects a wide range of vertebrate hosts, including mammals, birds, reptiles, and amphibians. *Giardia* comprises of six species according to the morphology and ultrastructure of their trophozoites: *Giardia agilis, G. ardeae, G. duodenalis, G. microti, G. muris, and G. psittaci*. However, *G. duodenalis* (syn. *G. intestinalis, G. lamblia*) is the only species found in humans and also in other mammals, including pets and livestock (Hunter and Thompson, 2005).

Giadiasis, one of the most frequently diagnosed intestinal protozoa infection, was reported globally. Approximately 200 million people in Asia, Africa and Latin America suffer from symptomatic giardiasis, with some 500,000 new cases each year (Thompson 2004). Prevalence of *G. duodenalis* is found in all age groups but children are at greatest risk for contracting clinical giardiasis especially those attending day care centers, living in poor hygienic conditions or in community settings (Mungthin *et al*., 2001, Saksirisampant *et al*., 2003). In young children, *G. duodenalis* causes diarrhea and impairs gain of body weight which interferes with growth and development (Berkman *et al*., 2002). Clinical manifestations of *G. duodenalis* infection vary from asymptomatic infection to chronic diarrhea, however, asymptomatic infection is usually observed. Host immunity plays an important role to control the disease. In Thailand, the prevalences of giardiasis studied in different groups of population varied from 1.3 to 37.7% (Wongsittwilairoong *et al*., 2007). Treatment alone is not the only implement to control the infection since re-infection is always observed. Understanding the epidemiology of *G. duodenalis* can give a significant

impact on developing methods of control and prevention, especially those of high risk groups.

Diagnosis of *Giardia* is based on light microscopic method. Using molecular tools such as the Polymerase Chain Reaction (PCR) with specific genes, genetic diversity within *G. duodenalis* have been demonstrated. The ability to accurately genotype *Giardia* not only determines sources of infection in outbreak situations but also provides direct evidence of zoonotic transmission. In addition, understanding the genetic variation of *G. duodenalis* among different populations will be useful in exploring the source of infection and transmission. Several specific genes have been studied for genotypic characterization of *G. duodenalis* i.e. SSU-rDNA, elongation factor 1-alfa (*ef1-α*), triose phosphate isomerase (*tpi*), b-giardin, glutamate dehydrogenase (*gdh*). Using these house keeping genes, *G. duodenalis* was differentiated into at least seven distinct genotypic clusters or assemblages (A to G) (Monis et al. 1999). Assemblages A and B isolates have been recovered from a broad range of hosts, including humans, livestock, cats, dogs, beavers and guinea pigs. Assemblages C and D are reported in dogs and cats while Assemblage E can be detected in cattle, sheep and goats. Others are Assemblages F and G which are found in cats and rats, respectively. All human *Giardia* isolates characterized to date, have been grouped into either Assemblage A or B, whereas, those isolates from cats, dogs and livestock have been identified either their own host specific assemblage or the potentially zoonotic Assemblages A and B. It has been demonstrated that analysis of only one gene locus using PCR-RFLP of the *gdh* gene, using 2 endonuclease enzymes, could identify Assemblage A into subgroups AI and AII, including Assemblage B into subgroups BIII and BIV(5). In contrast to other gene loci, genetic characterization of *G. duodenalis* into subgroups required at least 2 gene loci. Thus, the advantage of the *gdh*

gene to differentiate assemblages and subgroups of *G. duodenalis* can be simply performed without nucleotide sequencing. However, compared with other genes, amplification the DNA of *G. duodenalis* cysts in stool specimens using the *gdh* gene had detection limits when a small number of cysts presented in stools (Nunthavisai *et al*., 2007).

The wide prevalence and varied genotypic characterization of *Giardia* have led to ongoing research. In Thailand, most studies investigated the prevalences of giardiasis which have limited epidemiological value. This study will provide information of genotypic characterization of *G. duodenalis* including its epidemiological information among different groups of Thai population. Moreover, risk association for *Giardia* infection was also determined using the standardized questionnaires.

2. MATERIALS AND METHODS

2.1 Study population and study design

Population 1: pre-school and school children, suburban area in Ayuthaya province

A cross-sectional study of intestinal parasitic infections emphasized on *Giardia* was performed at a school, suburban area in Ayuthaya province, central Thailand, in 2005. A total of 429 preschool and school children, aged between 3-15 years old, were enrolled into the study.

Population 2: Hilltribe children, Chiangmai province, North Thailand

Between November 2006 and April 2007, following a protocol approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, a total of 40 positive *G. duodenalis* archived stool specimens were used in this study. Specimens were collected from a survey of intestinal parasitic infections, performed in hilltribe children of 2 districts at mountainous area, Mae Chaem and Hod districts, Chiangmai

province, Northern Thailand. Each fecal sample was recorded for the stool consistency (form, soft, mushy, and watery) by one laboratory technician throughout the study. The intensity of *Giardia* infection was performed using the average number of cyst in 10 high power fields in simple wet preparation under the light microscopy (40X). The scoring protocol was as followed: 1-2 cysts = + (very low), 3-10 cysts = ++ (low), 10-30 cysts = +++ (medium), and > 30 = ++++ (high), respectively. The purified *Giardia* cysts were washed thrice by phosphate buffered saline (PBS) and then kept frozen at -20^0C for genotyping study.

From the record, children were from 5 tribes such as Karen, Mong, Lava, Land and Lesor villages. The information about demographic data i.e. age, sex, tribe, weight, height from infected and uninfected children were used for data analysis.

Population 3: Preschool children in a kindergarten school, Takradan subdistrict, Sanamchaiket district, a rural area of Chacherngsao province, central Thailand

A cross-sectional study was performed during February 2007 in 225 children aged between 3-6.years at a kindergarten school, Takradan subdistrict, Sanamchaiket district, Chacherngsao province, central Thailand. There were 2 groups of children, pre-kindergarten (3-5 years old) and kindergarten (>5 years old)

Population 5: Orhans at Pak Kred babies' home, Nonthaburi province, central Thailand

A prospective cohort study was conducted for *G. duodenalis* infection in an orphanage (Pakred Babies' Home) located at Pakred district, Nonthaburi province during January 2007 to January 2008**.** Both orphans and childcare workers were enrolled into the study. There were 13 separate rooms provided for the orphans in this orphanage. Specific age groups of orphans were assigned to live in13 rooms, i.e. age newborn-8 months (Room no.2, 3 and 7), 9-16 months (Room no. 8 and 9), 7-24

months (Room no. 10), 25-32 months (Room no.6), 33-40 months (Room no.5), and more than 41 months (Room no. 1). Room no. 4 was assigned for positive-HIV infected orphans. Each room accommodated 20 to 50 orphans with 3 childcare workers. The childcare workers of each room were asked to complete the standardized questionnaires of their responsible orphans. Stool samples were collected every 4 consecutive months. The information including age, sex, weight, height, HIV status, the date of stool sample collected and the present illness (i.e. fever, nausea, diarrhea, abdominal pain) were collected.

Ethical clearance

The study for each population mentioned above was approved by the Ethical Committee of the Royal Thai Army, Medical Department. Informed consents were obtained from participants or parents or director of the babies' home before the enrollment, depended on the study population and design. Enrolled participants who were infected with pathogenic intestinal parasites were treated with appropriate antiparasitic drugs.

2.2 Stool examination. Stool samples were examined for the intestinal parasitic infections by simple wet preparation. Stool samples were concentrated using water-ethyl acetate sedimentation. Approximately 0.5 g stool sample was homogenized in 8 ml of distilled water and filtered through two layers of gauze. Three milliliter of ethyl acetate was added, vortexed for 1 min and then centrifuged at 700g for 2 min. After decanting the supernatant, the sediment was used for DNA preparation.

In addition, identification of *B. hominis* was performed using Jones' medium supplement with 10% horse serum and incubated at 37^0C for 2-3 days (Jones 1946, Leelayoova *et al*., 2002, 2009). *Cryptosporidium* and Microsporidia were also identified using modified-acid fast and gram-chromotrope staining, respectively.

Positive *Giardia* samples were collected using sodium nitrate floatation technique, washed three times with PBS and kept at -20^{0}C until used.

2.4 DNA extraction. Seven µl of each concentrated stool was applied onto both sides of a 6 mm-diameter FTA disk (Whatman, Bioscience, U.S.A.) in a 1.5 ml microcentrifuge tube and the FTA disk was dried overnight. The disk was cut into 4 pieces and 1 piece was used in one test. The FTA disk was washed twice with 200 µl of FTA purification buffer (Life technologies, Gaitherburg, MD.) for 15 min, then washed twice with 200 µl of TE buffer (10 mM Tris-HCl pH 8.0; 0.1 mM EDTA pH 8.0) for 5 min and dried overnight. Additionally, *Giardia* DNA was extracted using QIAmp stool mini kit (Qaigen, Germany) according to manufacturer's instructions. To concentrate the DNA, final elutions of DNA were made in 100 µl of elution buffer instead of 200 µl as recommended by the manufacturer.

2.5 Genotypic characterization. Genotypic characterization of *G. duodenalis* was determined by polymorphic sites using nested PCR of a 432 bp region of the glutamate dehydrogenase (*gdh*) gene using the condition as previously described (manuscript submitted). Briefly, primary amplification of *gdh* gene was performed using forward primers (GDH1, GDHeF) and reverse primer (GDHiR). The secondary pair of primers was GDHeF/GDHiR . A total mixture of 50 µl contained DNA template using a piece of FTA filter paper, 1x PCR buffer, 1.0 U of *taq* polymerase, 1.5 mM $MgCl_2$, 0.2 mM dNTP and 25 pmol of each primer. The PCR condition was as follow: 1 cycle of 94 °C for 2 min, 56 °C for 1 min and 72°C for 2 min, followed by 55 cycles of 94 °C for 30 s, 56 °C for 20 s and 72 °C for 45 s and a final extension of 72 °C for 7 min. RFLP analysis was performed by digesting 10 µl of the PCR product with 5 U of Nla*IV* in 1X enzyme buffer (New England Biolabs, England) in a final volume of 20 µl for 3 h at 37 °C. PCR products and restriction fragments were separated by electrophoresis in 2 %

agarose gel, respectively. Gels were stained with ethidium bromide and visualized under UV light and documented on high-density printing paper by using a UV–save gel documentation system I (UVItech, Cambridge, United Kingdom).

In addition, *Giardia* genomic DNA from fecal specimens which gave negative PCR results after using the *gdh* gene, was also amplified using small subunit-rRNA gene (SSU-rRNA). The amplification of a 130 bp fragment of *G. duodenalis* SSU-rRNA gene was carried out using nested PCR with the condition previously described by Hopkins *et al*.(1997): initial denaturing at 96 ºC for 2 min; then 35 cycles, each comprising denature at 96 ºC for 20 sec, annealing at 59 ºC for 20 sec, extension at 72 ºC for 30 sec followed by a final extension at 72 ºC for 7 min.

DNA sequencing of all PCR-positive samples were also performed to confirm with the sequence of GenBank accession number; L40509 (*G. duodenalis* assemblage AI), L40510 (*G. duodenalis* assemblage AII), AF069059 (*G. duodenalis* assemblage BIII), and L40508 (*G. duodenalis* assemblage BIV). Multiple alignment and restriction map analysis were performed using program Bioedit version 7.

2.6 Questionnaires. To determine the risk factors and outcomes of *G. duodenalis* infection, standardized questionnaires were used. Enrolled participants or their parents answered the questionnaires, covering demographic data, sanitary behaviors, sources and treatment method of drinking water, pets or animals contact including clinical symptoms.

2.7 Statistical analysis. The association between potential risk factors and *G. duodenalis* infection was assessed by the chi-square test with 95% confidence interval. For the cross-sectional study, univariate and multivariate regression analysis was performed to determine independent risk factors of *G. duodenalis* infection. For the cohort study in the orphan group, univariate and multivariate Poisson regression

analysis was performed using STATA 9.2 program to determine independent risk factors of *G. duodenalis* infection.

RESULTS

Population 1: Pre-school and Primary School Children, Ayuthaya Province, Central Thailand

Overall prevalence of enteroparasite infections in preschool and primary school children was 19.1%. The prevalence of each intestinal parasite is shown in Table 2. *Blastocystis hominis* (17, 7.9%) was the most predominant intestinal protozoa, and followed by *G. duodenalis* (13, 3.0%). Of 13 stool samples, 12 were successfully genotyped, which were genotype A 9/12 (75%), and genotype B 3/12 (25%), Table 1. As shown in Table 3, the prevalence of *G. duodenalis* infection was not significantly different regarding sex, age, classroom, numbers of children <5 years old lived in the same household, parents' education, have dogs or cats at home, history of animal contact (dogs or cats), wash hands before meal, type of drinking water and swimming in the pool. Statistically analysis showed that children infected with *G. duodenalis* did not significantly have diarrhea, abdominal pain, nausea, and weight loss (P>0.05). However, Fisher's exact test showed that *G. duodenalis* infected children was significantly associated with mucus stools (P =0.000) and WBC in stools (P =0.000) (T able 3). Although those with assemblage A reported mucus stool 5/8 (62.5%), not with assemblage B 0/3 (0%), there were no statistically significant associated with assemblages.

Population 2: Hilltribe Children, Mae Chaem and Hod districts, Chiangmai, Northern Thailand

The data composed of 765 school children , 589 from Mae-jam district and 176 from Hod district. The children were Karen (88.4%), Mong (6.7%), Lava (3.3%),

Lesor (1.0%) and Land (0.7%). The mean age was 8.15± 3.171 years (range, 1-16 years). The prevalences of *Giardia* infection in children were 5.1 % (30 in 589) from Mae-Chaem district and 5.7 % (10 in 176) from Hod district, respectively. Characteristics of infected children in the 2 districts are shown in Table 4. *Giardia* infection was found only in Karen (100%) in both groups. Other tribes showed negative *Giardia* infection.

Of 40 positive *G. duodenalis* samples, 28 (70%) were available for genetic characterization. Sequence analysis of SSU-rRNA gene revealed 57.1 % assemblage A, followed by assemblage B (42%). As shown in table 1, assemblage A (72.2%) was the most prevalent in infected children from Mae Chaem district while assemblage B (70%) was the highest in those from Hod district. Subgenotypes BIV and AII were common among these 2 groups of children, followed by subgenotype AI. Subgenotype BIII was found in a Karen child from Hod district

No significant differences between assemblage A and B infection were seen in sex and age group (Table 5). Fisher's exact test revealed that children infected with assemblage A were associated with loose to watery stools than those infected with assemblage B (p=0.001). Additionally, assemblage B had higher cyst shedding, 3-10 cysts/HPF or more, than those of assemblage A (p=0.019).

Population 3: Children at a kindergarten school, Takradan subdistrict, Sanamchaiket district, Chacherngsao province, central Thailand

Of 225 preschool children, 188 (83.6%) were voluntarily enrolled into the study. The prevalences of parasitic infections in the study population are shown in Table 6. The highest prevalence of *B. hominis* (8.3%).was found in this study. *G. duodenalis* was the second most common, with the prevalence of 4.8%. Other common intestinal parasites reported in these children were hookworm, *Strongylodes stercoralis*,

Enterobius vermicularis and *Trichomonas hominis*. *Cryptosporidium* spp., and microsporidia, were not found.

Characteristics of enrolled preschool children are shown in Table7. The prevalences of giardiasis were not significantly different between groups consisting of differences in age, sex, class, number of children aged <5 years lived in the same household, have dogs and cats at home, history of dog contact, wash hand before meal, drinking boiled water and source of drinking water. Univariate analysis showed that children who had cats in their houses were associated with *G. duodenalis* infection (*P* =0.033, 95% CI= 1.12-15.52). After adjusting for sex, age group, wash hands before meals, multivariate analysis confirm the association between children who had cats in their houses and giardiasis. Children who had cats in their houses were 5.1 times (P=0.021, 95% CI= 1.28-20.32) at greater risk of acquiring *Giardia* infection. (Table 8).

Population 4: Orhans at Pakred babies' home, Nonthaburi province, central Thailand

A total number of 895 stool specimens from 647 enrolled participants, comprised 381 males and 266 females, aged ranged from 1 month to 12 years. *G. duodenalis* was the second common protozoal infection (5.9%), after *B. hominis* infection (6.7%) (Table 9). Characteristics of *G. duodenalis* infected orphans were shown in Table10. There was one infected orphans who was reinfected during the study. One *G. duodenalis* infected orphan (0.3%) was HIV-positive. One of the childcare workers was found positive (0.3%). The highest prevalence was 2.5% in orphans aged between 37-48 months old, 1.4%were found in 49-60 months old, and 1.1% at the age of 25-36 months old, followed with 0.6% at the age of >60 months old respectively. In other age groups (0-12, 13-24 months old), 0% infected orphans were observed. The prevalences of *G. duodenalis* infection were significantly different

among different age groups (P<0.001). The high prevalences of *G. duodenalis* infection were found in room No.5 (8, 2.2%), No.6 (7, 2.0%), No.1 (2, 0.6%), respectively. In addition, the prevalences of *G. duodenalis* infection among rooms were significantly different (P<0.001). Most infected orphans had no gastrointestinal symptoms during the study. The prevalences of *G. duodenalis* declined gradually from January 2007 to January 2008 which were 5%, 2%, and 1%, respectively.

Assemblage A (78.6%) was the most prevalent found in orphans. The incidence of *Giardia* infection in orphans in room no. 5 and 6 was 0.0179 person-month compared the other rooms, 0.0015 person-month (95%CI =1.71-131.57). The univariate and multivariate analyses of risk factors associated with *G. duodenalis* infection are shown in Table 12. Univariate analysis showed that orphans aged between 25-48 years who lived in room No.5 and 6 had greater risk of acquiring *G. duodenalis* infection. There was no significant association between *G. duodenalis* infection with sex and diarrhea status. After adjusted for age range, sex, the multivariate analysis showed that those who lived in room No. 5 and 6 had 11.11 times (95% CI = 2.02-61.01) at greater risk of getting the infection than those who lived in other rooms.

DISCUSSION

The prevalence of *G. duodenalis* infection has been studied in several groups of population in different regions of Thailand during 1978-2007, such as orphans, school children, and adults with a wide range of 1.3% to 37.7% (Wongsittwilairoong *et al*., 2007). The prevalence in school children from low socioeconomic status was 21 % (Chavalittamrong and Jirapinyo1984). Prevalence surveys in orphans in 1990 and 2001 were 20 % and 12 %, respectively (Janoff *et al*., 1990, Mungthin *et al*., 2001). In addition, study of giadiasis in military personnel, mainly at 20 years of age, was 1-3 %

(Taamasri *et al* 2002, Leelayoova *et al*., 2009). The highest prevalence was 37.7% in an orphanage, Pathumthani province (Saksirisampant *et al*., 2003). Since the standard methods were used to detect *G. duodenalis* in stool specimens, we then compared our results to the previously reported studies. In our study, the prevalences of *G. duodenalis* infection in pre-school children (a kindergarten school, Sanamchaiket district, Chacherngsao Province), school children (Bangsai, Ayuthaya province), the Karen children (Mae Chaem and Hod districts, Chiangmai province), and orphans (Pak Kred Babies' home, Nonthaburi province) were in a range of 3-6.2% which had similar level as those reports in other regions of Thailand. The overall prevalence of *G. duodenalis* was not very high, compared to *B. hominis* (6.7-17.7%), a common intestinal protozoa infection which was diagnosed using the sensitive culture technique (Leelayoova *et al*., 2002). Thus, the true prevalence of *G. duodenalis* could be underestimated since laboratory diagnosis was based on microscopy (wet simple smear and concentration techniques) rather than other sensitive techniques such as ELISA and PCR method (Haque *et al*., 2005). Our recent published work also showed the benefit of PCR method using SSU-rRNA gene to detect *Giardia* cysts in stool specimens (Nunthavisai *et al*., 2007). If screening had been performed, higher prevalence of *G.duodenalis* could have been detected. The PCR-RFLP of the *gdh* gene is useful for discriminating assemblages into subgenotypes of *G.duodenalis*, however, a disadvantage of its limitation to detect small number of cysts has been observed. Thus, we conducted the molecular studies of *G.duodenalis* using both the *gdh* and SSU-rRNA genes in this study.

The predominant of *Giardia* assemblages was varied considerably from country to country depended on study population. In Mexico, Colombia, and Peru, assemblage A was the predominant group reported in children (Cedillo-Rivera *et al*., 2003, Ravid *et*

al 2007, Peréz Cordón *et al*., 2008., Minvielle *et al*., 2008). In Bangladesh, Philipines, Brazil, Egypt, Spain and Argentina, assemblage B was the most predominant (Haque *et al*., 2005, Yason and Rivera 2007, Kohli *et al*., 2008, Sahagun *et al*., 2008, Foronda *et al*., 2008). Equal distribution of assemblage A and B was found in Albania and Cuba (Berrilli *et al* 2006, Pelayo *et al*., 2008). Our results showed that geographical variation also occurred within Thailand. As shown in Table 1, the predominant of either assemblage A or B could occur, depended on the study population and study area. A high prevalence of assemblage A was found in hilltribe children from Mae Chaem district while assemblage B was the most prevalent in those from Hod district. Orhans had the highest prevalence of assemblage A. Additionally, the different predominant assemblage was found in study population of the same age range. Children at Chacherngsao province mainly harbored assemblage B while those at Ayuthaya province harbored assemblage A. Significant risk factors associated with *Giardia* infection may account for this finding since each population exposed to different exposures. Study of risk factors in epidemiological study could help answer the difference in assemblage finding in each population.

Our study in hilltribe children showed that 100% of *Giardia* infection were found among the Karen children from 2 districts, which none (0%) was found in other tribes (Lesor, Lava, Mong, and Land). Children from Mae Chaem had assemblage A as the most prevalent. In contrast to those from Hod district, assemblage B was the most predominant. Subgenotype BIII was noticed in 1 child, aged 8 years old from Hod district. Subgenotype BIII was an uncommon genotype reported in human infection which the infection might have come from animal sources. In this study, the Karen children who were infected with assemblage B had higher number of cyst shedding than those infected with assemblage A. Our results agree with the recent study that

children infected with assemblage B demonstrated greater number of cyst shedding (Kohli *et al*, 2008) and higher amount of genomic DNA of assemblage B detected in stool samples (Haque *et al*., 2005). A large number of cysts of assemblage B excreted in stools might help spreading the infective cysts which contaminated into the environment (Kohli *et al*, 2008). With these results, they hypothesized that a higher number of cyst shedding by assemblage B could be account for its high prevalence reported in population of some geographical areas having improper sanitation and contaminated drinking water.

A longitudinal study in the orphanage, Pakred's babies' home, showed that assemblage A, subgenotype AI was the most prevalent found in orphans. Multivariate analysis confirmed that orphans in room number 5 and 6 had a high risk of getting the infection. Those who lived in room number 5 and 6 were orphans aged 25-48 years, hence they had 11 times greater risk of getting *Giardia* infection than those lived in other rooms.. Person-to person transmission was the most likely to occur in orphans between this age group, since their personal hygiene such as toilet training, proper hand washing were not being well-trained. A chance of having infection by close physical contact with other infected ones was higher than those in the room with no infection or less infection rate. Subgenotype AI was found in 8/11, which could play a role for the transmission in this orphanage. Benefit of continue receiving the treatment after knowing results of positive stools was shown in this study, the prevalence of *Giardia* infection in orphans gradually decreased from 5% in January 2007, 2% in May 2007 and to 1% in January 2008, respectively. We showed how epidemiological study of *Giardia* infection in the orphanage had brought an effective prevention and control strategies to these orphans. As shown in this cohort study, when more attention was

paid to those high risk groups, the incidence or new cases could be significantly reduced and then the source of transmission could be further eliminated.

Risk factors for acquiring *Giardia* infection of primary school children in a rural community, Chacherngsao province, Thailand has been shown in our recent study (Ratanapo *et al*., 2008). Those significant risks were as followed; children of age 5-9 years, households with $\geq$ 3 children under the age of 12 years, low parental educational level, drinking bottle water, and living in close contact with dogs. In this study, further study of risk association was assessed in pre-school children, aged between 3-6 years old, in a kindergarten school in the same community. Univariate and multivariate analysis showed that having cats in the house was a significant risk for getting *Giardia* infection. Thus, multiple modes of transmission including zoonotic transmission of *G. duodenalis* from domestic animals, especially from dogs and cats, were suggested in this population. Direct evidence was not proved in this study since we did not examine stools of dogs and cats. However, a highly significant association between the prevalence of *Giardia* in humans in tea-growing community in India and presence of a Giardia-positive dog in the same household was demonstrated (Traub *et al* 2004). In Italy, surveys in stray and owned cats also revealed a number of cats harbored *G. duodenalis* assemblage A (37%) (Papini *et al*., 2006). Further study of *Giardia* infection in cats in our study community should be performed. Using tools in molecular epidemiology, our study provide information of significant risk factors associated with *Giardia* infection which could lead to the effective prevention and control programme of giardiasis including other protozoal infections in this community.

G. duodenalis infection cause asymptomatic and symptomatic infection in humans. In symptomatic cases, gastrointestinal illness are presented i.e. abdominal pain, nausea, vomiting, diarrhea, weakness, malnutrition, and stunt of growth in

children. Host factors such as nutritional and immunological status have played an important role and may affect these clinical outcomes of giardiasis (Cedillo-Rivera *et al*., 2003, Sahagun *et al*., 2008). The relation between clinical symptoms and the *Giardia* isolate genotype was also shown in many previous studies with the controversy outcomes. Homan and Mank (2001) studied in Dutch patients and observed a correlation between assemblage A and intermittent diarrhea, while assemblage B was presented in patients with persistent diarrhea. A study of *Giardia* infection in children in Peru showed that diarrheic children were associated only with assemblage A (Peréz Cordón *et al*., 2008). Sahagun *et al* (2008) also demonstrated a correlation between assemblage AII and symptomatic infections in children, specifically for the age under 5 years old. Additionally, a case-control study performed in Bangladesh showed that assemblage A and AII infections had higher odds ratios for diarrhea (Haque *et al*., 2005). We also observed a statistically significant difference of assemblage A causing loose or watery stools in the Karen children, Chiangmai province. Additionally, the results of infected school chidren at Ayuthaya province also showed that 6/9 (66.7%) school children who harbored assemblage A, had mucous stool while 3/3 (0%) school children infected with assemblage B showed non-mucous stool. However, there was no statistically significant difference between assemblage A and B as regard the appearance of mucous in stool. This might be due to small number of case Controversial results of the clinical presentation of giardiasis and their genotypes have been reported. A study in Cuban children demonstrated that assemblage B was more likely to cause symptomatic infection than children infected with assemblage A (Pelayo *et al*., 2008). However, the study in children in Mexico, Brazil, and Egypt exhibited no difference between *G. duodenalis* assemblage A and B with the clinical symptoms (Cedillo-Rivera *et al*., 2003, Kohli *et al*., 2008, Abdel-Moneim *et. al*., 2008). In this

study, we observed that about 10% of infected children had diarrhea. Host factors, especially the host immunity, account for these different findings of the clinical symptoms. Additionally, good nutrition and food supplement might be benefit to overcome or reduce symptoms caused by giardiasis. Thus, further investigations in different groups of population are needed to make a definite conclusion of these contrary results.

CONCLUSION

1. *Giardia* was the second most common protozoal infection among children. Using microscopic examination, the prevalences of *G. duodenalis* among children in different areas of study in Thailand were in a range of 3-6.2%. The highest prevalence was found in primary school children (6.2%).

2. The predominant assemblages could be either assemblages A or B depended on the study population. This could be related to different risk factors such as the source of transmission in each area of study. Thus, it could be person-to-person, waterborne, or zoonotic transmission.

3. Univariate and multivariate analysis showed that having cats in the house was a significant risk factor for getting *Giardia* infection in preschool children at Sanamchaiket district, Chacherngsao province. Zoonotic transmission could play a part in transmission cycle in this community.

4. Univariate and multivariate analysis showed that orphans lived in room no 5 and room no 6 had 11 times greater risk of getting *Giardia* infection than orphans in other rooms. As the results, orphans age 25-48 years old were a high risk group in this orphanage. Subgenotype AI could play an important role for person to person transmission in this orphanage.

5. In the Karen children, Assemblage A was significantly related to loose or watery stools. Additionally, Assemblage B had significantly higher number of cyst shedding in stool specimens. This might help spreading a large number of cysts of assemblage B, contaminated into the environment.

6. Diarrhea was found in 10% of the study population, thus, most cases were asymptomatic. Mucous stools were observed in the children ($P<0.05$). However, there was no statistically significant difference between assemblage A and B as regard the appearance of mucous in stool ($P>0.05$).

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Table 1 Summary of the prevalences of *G. duodenalis* assemblages A and B in 5 groups of study population

Study population/ area	Prevalence (%)	Assemblage A (%)	Assemblage B (%)
1. Preschool and school children			
Bangsai, Ayuthaya	3 % (13 /429)	**75.0 %** (9/12)	25.0%(3/12)
		A(5), AII (4)	B (3)
2. Hilltribe children	5.1% (30/589)	**72.2 %** (13/18)	27.8% (5/18)
Mae Chaem district, Chiangmai		AII (7)	BIV(2)
Hilltribe children	5.7 % (10/176)	30% (3/10)	**70%** (7/10)
Hod district, Chiangmai		AI(2), AII (1)	BIV(6)
			BIII(1)
3.Preschool children	4.8% (11/188)	30% (3/10)	**70%** (7/10)
Sanamchaiket district		AII (3)	BIV (6)
Chacherngsao			BIII (1)
4 *Primary school Children,	6.2% (33/531)	41.7%(5/12)	**58.3%** (7/12)
Sanamchaiket district		AII (5)	BIV (7)
Chacherngsao			
5. Orphans, Pak Kred babies'	5.9% (21/353)	**78.6%** (11/14)	21.4% (3/14)
home, Nonthaburi		AI (8)	BIV (3)
		AII (3)	

* Ratanapo et al., *Am J Trop Med Hyg* 2008; 78: 611-615.

Table 2 Intestinal parasitic infections in 429 school children, Ayuthaya province, central Thailand

Intestinal parasitic infection	Number	percent
Blastocystis spp.	76	17.7
Giardia duodenalis	13	3.0
Enterobius vermicularis	3	0.7
Opisthorchis viverrini	2	0.5
Hookworm	1	0.2
Cryptosporidium	0	0
Microsporidia	0	0

Table 3 Characteristics of 423 school children infected with *G. duodenalis*, Ayuthaya province, central Thailand

Characteristic	*G. duodenalis*		*P*
	Negative(%)	Positive (%)	
Sex			
Male	197 (97.0)	6 (3.0)	
Female	213 (96.8)	7 (3.2)	1.000
Age (years)			
3-6	50 (96.2)	2 (3.8)	
7-10	142 (96.6)	5 (3.4)	0.453
>10	214 (97.7)	5 (2.3)	
Number of Children age <5 in the same household			
2 or less	350(97.0)	11(3.0)	
>2	20 (100)	0(0)	1.000
Have dogs at home			
No	104 (96.3)	5(3.7)	
Yes	276(97.2)	8(2.8)	0.546
Play with dogs			
No	105 (96.3)	4 (3.7)	
Yes	292 (97.0)	9 (3.0)	0.751
Have cats at home			
No	263 (96.3)	10 (3.7)	
Yes	129 (97.7)	3 (2.3)	0.560
Play with cats			
No	232 (96.3)	9(3.7)	
Yes	146(98.0)	3(2.0)	0.548
Wash hands before meal			
No	236 (97.1)	7(2.9)	
Yes	164(97.0)	5(3.0)	1.000
Type of drinking water			
Others	99(98.0)	2 (2.0)	
Boiled or filtered	296(96.4)	11 (3.6)	0.533
Weakness			
No	313 (97.2)	9 (2.8)	
Yes	64 (94.1)	4 (5.9)	0.254
Weight loss			
No	364(97.1)	31 (2.9)	
Yes	12 (92.3)	1 (7.7)	0.340
Diarrhea			
No	335 (96.5)	12 (3.5)	
Yes	45(97.8)	1(2.2)	1.000
Mucous in stool			
No	411(98.6)	6 (1.4)	
Yes	1 (12.5)	7 (87.5)	0.000*
WBC in stool (Microscopic Examination)			
No	409 (98.3)	7(1.7)	
Yes	7(53.8)	6 (46.2)	0.000*

Table 4 Characteristics of hilltribe children from 2 schools, positive with *G. duodenalis*, Mae Chaem district and Hod district, Chiangmai province, North Thailand

	Mae-jam school		Hod school	
		G. duodenalis		*G. duodenalis*
Characteristic	Number	Infection (%)	Number	Infection (%)
Sex				
Male	302 (51.3)	14 (4.6)	83 (47.4)	6 (7.2)
Female	287 (48.7)	16 (5.6)	92 (52.6)	4 (4.3)
Total	589 (100)	30 (5.1)	175 (100)	10 (5.7)
Age (years)				
1-3	65 (11.0)	3 (4.6)	12 (6.9)	0 (0)
4-6	89 (15.1)	4 (4.5)	45 (25.7)	5 (11.1)
7-9	264 (48.8)	15 (5.7)	33 (24.6)	2 (4.7)
10-12	145 (24.6)	3 (2.1)	31 (18.9)	2 (6.1)
>12	26 (4.4)	5 (19.2)	42 (24.0)	1(2.4)
Total	589 (100)	30 (5.1)	175 (100)	10 (5.7)
Tribe				
Karen	502 (85.2)	30 (6.0)	174 (98.9)	10 (5.8)
Mong	50 (8.5)	0 (0)	1 (0.6)	0 (0)
Lava	24 (4.1)	0 (0)	1 (0.6)	0 (0)
Lesor	8 (1.4)	0 (0)	0	0 (0)
Land	5 (0.8)	0 (0)	0	0 (0)
Total	589 (100)	30 (5.1)	176 (100)	10(5.7)

Table 5 Characteristics of the Karen children, Mae Chaem and Hod districts, infected with *G. duodenalis* assemblage A and B

Characteristic	Assemblage found in infected children		*P*
	A	B	
Sex			
Male	9 (64.3.3)	5 (35.7)	
Female	7 (50.0)	7 (50.0)	0.704
Age (years)			
3-10	12 (54.5)	10 (45.5)	
> 10	4 (66.7)	2 (33.3)	0.673
Cyst shedding in stool			
1-2 cysts/ HPF	10 (83.3)	2 (16.7)	
3-10 cyst or more/ HPF	5 (33.3)	10 (66.7)	0.019*
Stool consistency			
Form	1(11.1)	8 (88.9)	
Others	15(78..9)	4 (21.1)	0.001*
(Mushy/Mucous/ Watery)			

* Signicifant difference by Fisher's exact test

Table 6 Intestinal parasitic infections in preschool children, Takradan subdistrict, Sanamchaiket district, a rural area of Chacherngsao province, central Thailand

Intestinal parasitic infection	Number	percent
Blastocystis spp.	19	8.3
Giardia duodenalis	11	4.8
Hookworm	3	1.3
Strongylodes stercoralis	2	0.9
Enterobius vermicularis	1	0.4
Trichomonas hominis	1	0.4

Table 7 Univariate analysis of risk factors of *Giardia duodenalis* infection, preschool children in a kindergarten school, Takradan subdistrict, Sanamchaiket district, Chacherngsao province

Characteristics	*Giardia duodenalis*		Crude odds ratio (95%CI)	*P*-value
	Negative (%)	Positive (%)		
Age group (years)				
>5	57(96.6)	2(3.4)	1	
3-5	117(93.6)	8(6.4)	1.95(0.40-9.48)	0.505
Sex				
Female	90(95.7)	4(4.3)	1	
Male	88(92.6)	7(7.4)	1.79(0.51-6.33)	0.361
Class group				
Pre-Kindergarten	11(91.7)	1(8.3)	1	
Kindergarten	167(94.4)	10(5.6)	1.52(0.18-12.96)	0.524
No. of children age<5 years living in the same household				
>2	49(96.1)	2(3.9)	1	
<2	99(92.5)	8(7.5)	1.98(0.41-9.68)	0.502
Keeping dog(s) at home				
No	41(91.1)	4(8.9)	1	
Yes	109(94.8)	6(5.2)	0.56(0.15-2.10)	0.469
Close contact to dogs				
No	39(97.5)	1(2.5)	1	
Yes	107(92.2)	9(7.8)	3.28(0.40-26.74)	0.454
Having cat(s) at home				
No	111(96.5)	4(3.5)	1	
Yes	40(87.0)	6(13.0)	4.16(1.12-15.52)	0.033*
Close contact to cats				
No	75(96.2)	3(3.8)	1	
Yes	62(89.9)	7(10.1)	2.82(0.70-11.37)	0.190
Washing hands before meal				
Every time			1	
Occasionally	50(98.0)	1(2.0)	4.59(0.57-37.27)	0.169
Drinking boiled water	98(91.6)	9(8.4)		
Yes				
No	22(95.7)	1(4.3)	1	1.000
Source of drinking water	129(94.2)	8(5.8)	1.36(0.16-11.45)	
Water on tap/Bottled water				
Others	7(100.0)	0(0)	1	
	147(94.8)	8(5.2)	1.05(1.02-1.09)	1.000
Usage of ice cube				
Ice cube in sealed Plastic bags	18(94.7)	1(5.3)	1	
Others	123(93.2)	9(6.8)	1.32(0.16-11.02)	1.000

CI = confidence interval

*Significant difference at P< 0.05

Table 8 Multivariate analysis of risk factors of *G. duodenalis* infection in preschool children in a kindergarten school, Takradan subdistrict, Sanamchaiket district, Chacherngsao province

Characteristics	Crude odds ratio (95%CI)	Adjusted odds ratio (95%CI)	*P*-value
Age group (years)			
>5	1	1	
3-5	1.95(0.40-9.48)	0.46(0.08-2.53)	0.371
Sex			
Female	1	1	
Male	1.79(0.51-6.33)	0.46(0.11-1.92)	0.285
Having cat(s) at home			
No	1	1	
Yes	4.16(1.12-15.52)	5.10(1.28-20.32)	0.021*
Washing hands before meal			
Every time	1	1	
Occasionally	4.59(0.57-37.27)	0.25(0.03-2.14)	0.207

CI = confidence interval

* Significant difference at $p < 0.05$ after adjusted for age group, sex, washing hands before meal

Table 9 Intestinal parasitic infections in orphans, Orhans at Pak Kred Babies' Home, Nonthaburi province, central Thailand

Intestinal parasitic infection	Number	percent
Blastocystis spp.	24	6.7
Giardia duodenalis	21	5.9
Entamoeba coli	8	2.2
Endolimax nana	2	0.6
Opistorchis viverrini	0	0
Trichuri trichiura	0	0

Table 10 The characteristics of enrolled orphans with *G. duodenalis* infection, Pak Kred Babies' Home, Nonthaburi province

Characteristics	**No. positive for *G. duodenalis***	**Total**	**%**	***p*- value**
Age (months)				
0-12	0	1	0	
13-24	0	181	0	
25-36	4	72	1.1	
37-48	9	39	2.5	
49-60	5	22	1.4	
>60	2	39	0.6	<0.001*
Room				
No. 1	2	30	0.6	
No. 2	0	34	0	
No. 3	1	50	0.3	
No. 4	1	68	0.3	
No. 5	8	25	2.2	
No. 6	7	27	2.0	
No. 7	0	35	0	
No. 8	0	32	0	
No. 9	1	28	0.3	
No. 10	1	28	0.3	<0.001*****
Sex				
Male	11	206	2.8	
Female	10	151	3.1	0.611
HIV infection				
No	20	299	5.6	
Yes	1	58	0.3	0.221
Diarrhea				
No	19	290	6.5	
Yes	1	3	0.3	0.192

* Significant difference at P <0.05

Table 11 Characteristics of orphans with *G. duodenalis* infection, Pak Kred Babies' Home, Nonthaburi province.

Characteristics	**January 2007**		**May 2007**		**January 2008**	
	No. (%)	***Giardia* infection (%)**	**No. (%)**	***Giardia* infection (%)**	**No. (%)**	***Giardia* infection (%)**
***Age (months)**						
0-12	87 (29.2)	0 (0)	84(27.9)	0 (0)	76 (26.0)	0 (0)
13-24	53 (17.8)	2 (0.7)	56(18.6)	1 (0.3)	46 (15.9)	0 (0)
25-36	33 (11.1)	6 (2.0)	26 (6.6)	3 (1.0)	22 (7.6)	0 (0)
37-48	20 (6.7)	4 (1.3)	24 (8.0)	2(0.7)	24 (8.3)	1 (0.3)
49-60	12 (4.0)	0 (0)	9 (3.0)	0 (0)	22 (7.6)	1 (0.3)
>60	93 (31.2)	3 (1.0)	102 (33.9)	0 (0)	100 (34.6)	1 (0.3)
Total	298(100)	15 (5.0)	301 (100)	6 (2.0)	289(100)	3(1.0)
***Room**						
No. 1	28 (9.7)	1 (0)	16 (5.6)	0 (0)	24 (8.3)	1 (0.3)
No. 2	20 (6.9)	0 (0)	21 (7.3)	0 (0)	18 (6.2)	0 (0)
No. 3	28 (9.7)	0 (0)	30 (10.5)	1(0.3)	30 (10.3)	0 (0)
No. 4	46 (15.9)	1 (0.3)	53 (18.5)	0 (0)	49(16.9)	0 (0)
No. 5	26 (9.0)	5 (1.7)	27 (9.4)	4 (1.4)	34 (11.7)	0 (0)
No. 6	30 (10.4)	9 (3.1)	24(8.4)	1 (0.3)	25 (8.6)	1 (0.3)
No. 7	23 (8.0)	0 (0)	28 (9.8)	0 (0)	20 (6.9)	0 (0)
No. 8	29 (10.0)	0 (0)	29 (10.1)	0 (0)	26 (9.0)	0 (0)
No. 9	31 (10.7)	0 (0)	28 (9.8)	0 (0)	24 (8.3)	0 (0)
No. 10	27 (9.3)	0 (0)	25 (8.7)	0 (0)	26(9.0)	1 (0.3)
No. 11	1 (0.3)	0 (0)	2 (0.7)	0 (0)	1 (0.3)	0 (0)
No. 12	-		4 (1.4)	0 (0)	1 (0.3)	0 (0)
No. 13	-				12 (4.1)	0 (0)
Total	289 (100)	16 (5.5)	287(100)	6 (2.1)	290(100)	3(1.0)
Sex						
Male	132 (43.7)	5 (1.7)	316 (45.2)	4 (1.3)	123 (42.6)	2(0.7)
Female	170 (56.3)	11 (3.6)	165 (54.8)	2 (0.7)	166(57.4)	1(0.3)
Sex						
Male	132 (43.7)	5 (1.7)	316 (45.2)	4 (1.3)	123 (42.6)	2(0.7)
Female	170 (56.3)	11 (3.6)	165 (54.8)	2 (0.7)	166(57.4)	1(0.3)
HIV infection						
No	189(81.1)	12(5.2)	175(79.9)	6(2.7)	170(80.6)	2(0.9)
Yes	44(18.9)	1(0.4)	44(21.1)	0(0)	41(19.4)	0(0)

*Significant difference among age group and room ($p<0.05$)

Table 12 Univariate and multivariate analysis of risk factors associated with *Giardia* infection in orphans, Pak Kred Babies' Home, Nonthaburi province

Characteristic	**No. positive for** *Giardia*	**Person-month of follow-up**	**Crude RR (95%CI)**	**Adjusted RR* (95%CI)**
Sex				
Female	1	0.0017	1	1
Male	5	0.0051	2.92(0.33-138.31)	2.36(0.27-20.31)
Room				
Others	2	0.0015	1	1
No.5 and 6	4	0.0179	11.90 (1.71-131.57)	11.11(2.02-61.01)*
Age (month)				
Others	2	0.0018	1	
25-48	4	0.0092	5.13(0.73-56.67)	

CI, confidence interval.
*Significant difference after adjusted for age group and sex

Improved Sensitivity of PCR amplification of Glutamate Dehydrogenase Gene for the detection of *Giardia duodenalis* in Stool Specimens

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Running title: PCR methods amplifying GDH gene of *G. duodenalis*

ABSTRACT

Herein we present the modified set of primers to increase the sensitivity of PCR amplification of glutamate dehydrogenase (*gdh*) gene to detect *Giardia duodenalis* cysts in stool specimens. Using nested-PCR, the modified set of primers had significantly higher sensitivity than that of previously published PCR primer set, 73.1% (95%CI 62.9-81.8) and 45.2 % (95%CI 34.8-55.8), respectively.

Giardia duodenalis is an intestinal flagellate protozoa that infects a wide range of hosts e.g., humans, livestock and domestic animals [1]. Molecular studies reveal that *G. duodenalis* is a complex species composed of genotypic distinctions [2-5]. Two major genetic assemblages i.e. A and B of *G. duodenalis* are recovered from humans [2, 3, 5]. Assemblage A has two distinct clusters; AI and AII while assemblage B consists of BIII and BIV [3]. Genotypic characterization of *G. duodenalis* has been shown to be a useful tool in epidemiological studies or outbreak investigations [6, 7]. PCR techniques for genotyping of *G. duodenalis* are based on the polymorphic nature of *G. duodenalis* DNA sequences of the small-subunit (SSU) rRNA, glutamate dehydrogenase (*gdh*), elongation factor 1-alpha (*ef1-α*), triose phosphate isomerase (*tpi*), and β-giardin [3, 4, 8, 9]. Using PCR amplification, all of these genes can differentiate subgenotypes of assemblage A into AI and AII. However, β-giardin gene and the highest amplified target of SSU-rRNA gene could not be used to discriminate the subgenotypes of assemblage B [10]. As the PCR methods for the detection of *gdh* gene can provide all information of *G. duodenalis* subgenotypes (AI, AII, BIII and BIV) [3, 12, 14], however, the sensitivity of PCR amplification of the *gdh* gene is rather limited in particular when low number of cysts presented in fecal samples [11, 13]. A few primer sets have been developed to amplify the *gdh* gene i.e., semi-nested PCR using GDHeF/GDHiR and GDHiF/GDHiR primers [14] and GDH1/GDH4 [12]. In our experiences, working on the GDHeF/GDHiR and the GDHiF/GDHiR primers to amplify the *gdh* gene using clinical specimens with light infection of *G. duodenalis* occasionally produced unsatisfied outcomes including negative results. Moreover, instead of obtaining the expected fragment at 435 bp, some nonspecific bands have been visualized (Fig 1A). Additionally, the PCR products sometimes showed a smear or fuzzy bands (Fig 1B),

of which interfered the desired band. Thus, this study aimed to increase the sensitivity of nested PCR of the *gdh* gene for the detection of *G. duodenalis* in fecal specimens. The GDH1 primer was used together with new modified primers, GDH1a and GDH5s, in primary PCR and compared the sensitivities to the published primers, GDH1 and GDH4 reported by Homan *et al.* [12]. Then, the secondary PCR was performed using the same set of primers GDHeF and GDHiR previously designed by Read *et al*. [14].

Non-preserved fecal specimens of *G. duodenalis* were obtained from survey studies of intestinal parasitic infections in an orphanage, schools and rural communities which have been approved by the ethical committee of the Royal Thai Army Medical Department. *Giardia* cysts were collected by sodium nitrate flotation technique. Briefly, stools were suspended in PBS, filtered through gauze. Approximately 2 g of stool were mixed thoroughly with saturated $NaNO_3$ and left for 20 min in a 15 ml centrifuge tube. Cysts at the top 1 ml of supernatant were collected from each sample, transferred to a 1.5 ml tube, and then washed three times with PBS. The final sediment was resuspended in PBS and kept at -20^{o}C until used.

Giardia DNA was extracted using FTA disk (Whatman, Bioscience, U.S.A.). Fifteen µl of each concentrated specimen was applied onto a 6 mm-diameter FTA disk which was allowed to air-dry overnight. The disk was cut into 4 pieces and 1 piece was used in one test. The FTA disk was washed twice with 200 µl of FTA purification buffer (Life technologies, Gaitherburg, MD.) for 15 min, then washed twice with 200 µl of TE buffer (10 mM Tris-HCl pH 8.0; 0.1 mM EDTA pH 8.0) for 5 min and dried overnight. The washed FTA disks were used as DNA templates in PCR amplification. In addition, QIAmp stool mini kit (Qiagen, Germany) was used

for *Giardia* DNA extraction in the fecal specimens giving negative PCR results after using FTA disk.

To design primers, sequence information of the *gdh* gene was obtained from the NCBI database; GenBank accession number: L40509 (assemblage AI) [3]; L40510 (assemblage AII) [3]; AF069059 (assemblage BIII) [4]; L40508, AY826191, AY826192, and AY826193 (assemblage BIV) [3, 7]; U60984 (assemblage C) [9]; U60986 (assemblage D) [9]; U47632, AY826198, AY826199, and AY826200 (assemblage E) [7, 9]. The sequences were aligned using ClustalW version 1.83 [15]. To amplify the *gdh* gene, a primary External Forward Primer, GDH1a (5'ATC TTC GAG AAG GAT GCT TGA G3') and External Reverse Primer, GDH5s (5'GGA TAC TTS TCC TTG AAC TC3') were developed using Primer3 (http://frodo.wi.mit.edu/primer3/input.htm). Modified primers were designed to cover all cognate sequences and to enable amplification of isolates across all assemblages. The primer GDH1a was designed based on primer GDH1 with one more adenine base inserted. This insertion was added according to the sequence variation at the priming position of primer GDH1 deposited in the genbank (AY826191, AY826192, AY826193, AY826198, AY826199, and AY826200). Using primer GDH1, together, with primer GDH1a will cover all variation existed at these positions. The GDH5s primer contained one degenerate base, S, where S = G+C. In a primary PCR amplification, primers GDH1, GDH1a and GDH5s spanned the *gdh* domain from base +138 to base +2461 which lead to the production of 2324 bp fragment. Compare to primers described by Homan *et al*. [12], the forward primer GDH1 (5'ATC TTC GAG AGG ATG CTT GAG3') and reverse primer GDH4 (5'AGT ACG CGA CGC TGG GAT ACT3') spanned the *gdh* domain from base +138 to base +907 and produced 770 bp fragment. By using the same set of primers in secondary PCR, a 461

bp of the *gdh* gene was amplified using GDHeF (5'TAC ACG TYA AYC GYG GYT TCC GT3') and GDHiR (5'GTT RTC CTT GCA CAT CTC C3') (Table 1). The restriction sizes of the bands digested with *Nla*IV and *Rsa*I were predicted by the restriction map using program ApE-A plasmid Editor version 1.11.

The first-round PCR amplification was performed using mixtures of 2 U of *Taq* polymerase with 1X PCR buffer, 2.5 mM $MgCl_2$, 250 mM of each dNTP, 25 pmol of each primer, and 2 pieces of FTA disks (or 1-2 µl of the extracted DNA) in a total volume of 50 µl. The thermal cycling conditions were as follow: 94^{o}C for 7 min and then 35 cycles of 94^{o}C for 1 min, 55^{o}C for 1 min, and 72^{o}C for 1 min, with a final cycle of 72^{o}C for 7 min. The second-round PCR was performed using mixtures of 2U of *Taq* polymerase with 1X PCR buffer, 1.5 mM $MgCl_2$, 200 mM of each dNTP, 25 pmol of each primer, and 1 µl of the primary PCR product in a total volume of 50 µl. The thermal cycling initiated with 1 cycle of 94°C for 2 min, 56°C for 1 min, and 72°C for 2 min, followed by 55 cycles of 94°C for 30 sec, 56°C for 20 sec, 72°C for 45 sec, and final extension at 72°C for 7 min. The PCR products were analyzed by 2% agarose gel electrophoresis, stained with ethidium bromide and then visualized on a UV transilluminator (Fig 2A and 2B).

A total of 93 *G. duodenalis*-positive specimens were analyzed by PCR using amplification of the *gdh* gene. Using GDH1/GDH4 primers and secondary primers of GDHeF/GDHiR, 42 (45.2%, 95%CI 34.8-55.8) were successfully amplified. Whereas, 68 (73.1%, 95%CI 62.9-81.8) were PCR positive using modified primary primers (GDH1/GDH1a/GDH5s) and secondary primers (GDHeF/GDHiR). The sensitivity of modified primer set (GDH1/GDH1a/GDH5s) was significantly higher than that of GDH1/GDH4 primers (chi-square, $p < 0.05$). Using GDHeF/GDHiR as secondary primers showed satisfactory results of clear bands. Beneficially, the

diagnostic genotyping profiles from the original article [14] still can be promptly used to analyze RFLP patterns.

In conclusion, nucleotide polymorphism of the *gdh* gene provides a rapid means to identify all subgenotypes of *G. duodenalis*. However, the use of the *gdh* primer for molecular identification requires a better primer pairs. This study provides the modified set of primers to improve the sensitivity of PCR amplification of the *gdh* gene of *G. duodenalis* in human fecal specimens.

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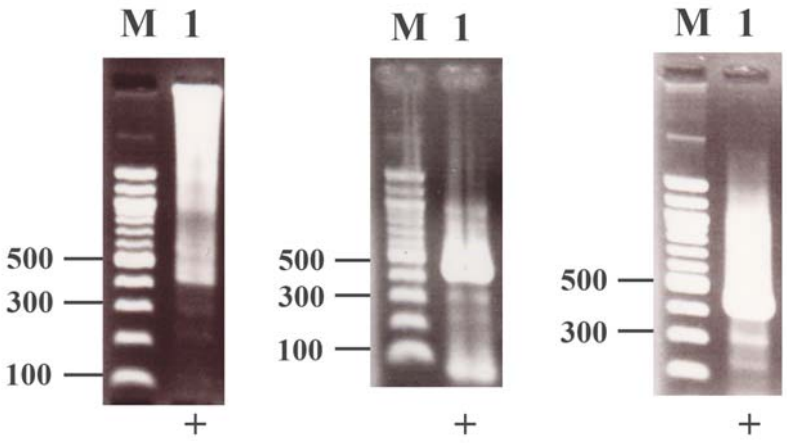
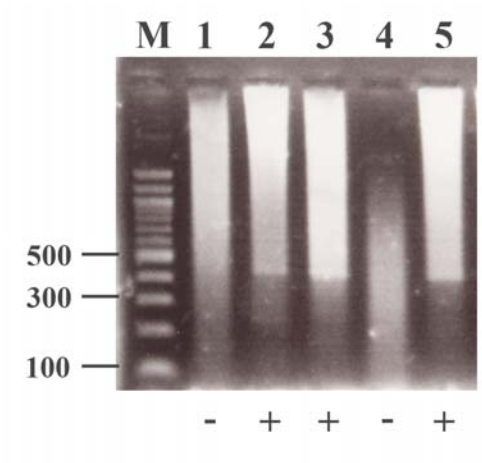
Table 1 Sequences of PCR primers of the *gdh* gene used for the detection of *G. duodenalis* cysts in this study.

PCR	Directions	Primers	Fragment sizes (bp)	References
Primary	Forward	GDH1 (5'ATC TTC GAG AGG ATG CTT GAG3')	2324	This study
	Forward	GDH1a (5'ATC TTC GAG AAG GAT GCT TGA G3')		
	Reverse	GDH5s (5'GGA TAC TTS TCC TTG AAC TC3')		
Secondary	Forward	GDHeF (5'TAC ACG TYA AYC GYG GYT TCC GT3')	461	Read *et al*. [14]
	Reverse	GDHiR (5'GTT RTC CTT GCA CAT CTC C3')		
Primary	Forward	GDH1 (5'ATC TTC GAG AGG ATG CTT GAG3')	770	Homan *et al.* [12]
	Reverse	GDH4 (5'AGT ACG CGA CGC TGG GAT ACT3')		
Secondary	Forward	GDHeF (5'TAC ACG TYA AYC GYG GYT TCC GT3')	461	Read *et al*. [14]
	Reverse	GDHiR (5'GTT RTC CTT GCA CAT CTC C3')		

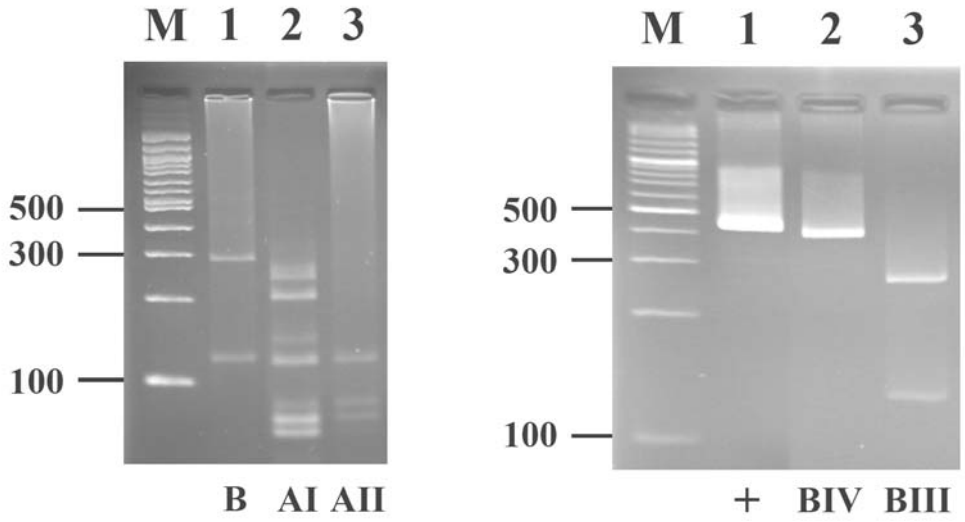


A



B

Figure 1 (A) Ethidium bromide stained 2% agarose gel showed three times amplification of the *G. duodenalis* positive controls at *gdh* gene using GDHeF/GDHiR as primary primers and GDHiF/GDHiR as secondary primers. The same amounts of DNA were used in all reactions. Lane M, 100 bp molecular weight marker; lane 1, positive controls. (B) The amplification of the faecal specimens at the *gdh* gene using GDHeF/GDHiR and GDHiF/GDHiR as primary primers and secondary primers, respectively; lane M, 100 bp molecular weight marker; lane 1 and 4 negative results for PCR; lane 2, 3, and 5, positive results for PCR.



A **B**

Figure 2 Ethidium bromide stained 2% agarose gel showed the PCR products amplified at the *gdh* gene of *G. duodenalis*, using GDH1/GDH1a/GDH5s and GDHeF/GDHiR as primary primers and secondary primers, respectively. (A) The PCR products were digested by enzyme *NlaIV*. Lane M, 100 bp molecular weight marker; lane 1, assemblage B; lane 2, assemblage A subgenotype AI; lane 3, assemblage A subgenotype AII. (B) The PCR products were digested by enzyme *RsaI*. Lane M, 100 bp molecular weight marker; lane 1 uncut PCR product; lane 2, assemblage B subgenotype BIV; lane 3, assemblage B subgenotype BIII.

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Evaluation of the Sensitivities of DNA Extraction and PCR Methods for Detection of *Giardia duodenalis* in Stool Specimens▽

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Sensitivities of DNA extraction methods and PCR methods for *Giardia duodenalis* were evaluated. A combination of the most sensitive methods, i.e., FTA filter paper and a PCR protocol using RH11/RH4 and GiarF/GiarR primers, showed no significant differences compared to immunofluorescence assay in terms of their sensitivities and specificities.

Giardia duodenalis is an intestinal flagellate that infects humans and other mammals, including pets and livestock, throughout the world. Approximately 2.8×10^8 people are infected by this organism each year (5). Light microscopy or immunofluorescence assay has been used to identify *G. duodenalis* in most laboratories. However, these techniques might not be sensitive enough to detect low numbers of excreted cysts (4). In addition, these methods cannot be used to differentiate genotypes of *G. duodenalis*. Recently, a few PCR-based techniques have been developed for detection and genotypic characterization of *G. duodenalis* (2, 3, 6, 9, 11). However, PCR techniques using stool specimens could be insensitive because of PCR inhibitors and the difficulty of cyst disruption. To raise the sensitivity of PCR, an effective DNA extraction method is needed. Commercial DNA extraction kits such as the QIAamp stool minikit (QIAGEN, Hilden, Germany) and FTA filter paper (Whatman Bioscience, Cambridge, United Kingdom) have been used for isolation of *Giardia* DNA (8, 12). However, these DNA extraction methods have never been compared. To determine the efficiencies of the three DNA extraction methods, i.e., FTA filter paper (Whatman Bioscience, United Kingdom), the QIAamp stool minikit (QIAGEN, Germany), and the conventional phenol-chloroform method, a known number of *G. duodenalis* cysts was used. Cysts of *G. duodenalis* were concentrated by saturated sodium nitrate flotation from a positive specimen collected from an asymptomatic member of the army during an annual health examination. The sample was washed three times with phosphate-buffered saline, followed by cyst counting using a hemocytometer. The sample was then 1:5 serially diluted to obtain 16,842, 3,368, and 674 cysts/ml. In addition, a 1:2 serial dilution of sample containing 674 cysts/ml was performed (to give 337 and 168 cysts/ml). These solutions containing different number of cysts were used for three DNA extraction methods. Since 200 μl of each dilution was used for DNA isolation by the QIAamp stool minikit and conventional phenol-chloroform methods and only 10 μl of each dilution was used as DNA template in PCR amplification, the numbers of cysts per PCR were 168, 34, 7, 3, and 2, respectively. In the FTA method, the amount of specimen placed on the FTA disk was limited to 15 μl, and only one-fourth of the FTA disk was used for each test. Thus, the numbers of cysts per PCR were equivalent to 63, 13, 3, 1.3, 0.6.

For DNA extraction with the FTA filter paper, the filter disk was allowed to air dry overnight after the application of specimens. The one-fourth piece of FTA disk was washed twice with 200 μl of FTA purification reagent (Life Technologies, Gaithersburg, MD) for 15 min and then washed twice again with 200 μl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) for 15 min and air dried overnight. The washed paper was then used directly as the DNA template in PCR amplification. For the QIAamp stool minikit (QIAGEN, Germany), 200 μl of each diluted sample was used for DNA extraction, following the manufacturer's instructions. The extracted DNA of each sample was kept frozen at −20°C until used. The phenol-chloroform extraction was performed as described by Hopkins et al. (3). The most efficient extraction method was defined as the method that could extract DNA from the lowest cyst numbers and that gave a positive band of *G. duodenalis* using the RH11/RH4-GiarF/GiarR primer set with the PCR conditions described by Hopkins et al. (3).

The comparison showed that FTA filter paper was the most efficient DNA extraction method; it could detect as few as 168 cysts/ml, while both the QIAamp stool minikit and phenol-chloroform extraction method could detect 674 cysts/ml stool dilution. In addition to its high sensitivity, the FTA filter paper assay was simple to use and can be applied with a large number of samples at one time. The samples are also easy to handle and transport for further analysis. The major disadvantage of using this procedure may be that some parts of the disk may contain more DNA template than other parts. This can affect the result of PCR amplification. To handle this problem, at least two PCR amplifications per disk of FTA filter paper are recommended.

PCR techniques for detection of *G. duodenalis* are based on the polymorphic nature of *G. duodenalis* DNA sequences of

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TABLE 1. Comparison of the sensitivities of five PCR methods for the detection of *G. duodenalis* trophozoite DNA and cysts in stool specimens

Primers	Target gene locus	Expected amplicon size (bp)	Reference	PCR result[a] for:									
				Trophozoite DNA concn (pg/μl) of:						Cyst concn (cysts/ml) of:			
				100,000	10,000	1,000	100	10	1	3,368	674	337	168
RH11/RH4 and GiarF/GiarR	SSU rRNA	130	3	+	+	+	+	+	−	+	+	+	+
G7/G759 and forward/reverse	β-Giardin	511	2	+	+	+	+	−	−	−	−	−	−
GDHeF/GDHiR and GDHiF	Glutamate dehydrogenase	432	9	+	+	+	−	−	−	+	+	+	−
AL3543/AL3546 and AL3544/AL3545	Triosephosphate isomerase	530	11	+	+	+	−	−	−	+	−	−	−
EF1AR/GLONGF and RTef1-αF/RTef1−αR	Elongation factor 1-alpha	191	6	+	+	−	−	−	−				

[a] All PCR amplifications were done in triplicate. +, at least one positive result out of three attempts.

the small-subunit (SSU) rRNA, glutamate dehydrogenase (GDH), elongation factor1-alpha (ef1-α), triosephosphate isomerase, and β-giardin genes (2, 3, 6, 9, 11). However, these PCR methods have never been compared. We determined the sensitivities of PCR primers with *G. duodenalis* trophozoite DNA as a template. Genomic DNA was extracted from assemblage B of *G. duodenalis* axenic trophozoites provided by the Department of Protozoology, Faculty of Tropical Medicine, Mahidol University, using the QIAamp stool minikit (QIAGEN, Germany). After DNA extraction, the concentration of the DNA sample was determined by UV absorption at 260 nm. *G. duodenalis* trophozoite DNA was then serially diluted 1:10 (to 100,000, 10,000, 1,000, 100, 10, and 1 pg/μl, respectively) and used for PCR amplification with different primer sets, i.e., RH11/RH4 and GiarF/GiarR (3), GDHeF/GDHiR and GDHiF (9), AL3543/AL3546 and AL3544/AL3545 (11), G7/G759 and forward/reverse (2), and EF1AR/GLONGF and RTef1-αF/RTef1-αR (6). Genomic DNA and the primer sets were used with previously described PCR conditions (2, 3, 6, 9, 11). The most sensitive primer pair was defined as the one that could amplify DNA of *G. duodenalis* at the lowest trophozoite DNA concentration. The results showed that RH11/RH4 and GiarF/GiarR primer set, amplifying the SSU rRNA gene, was the most sensitive primer set and could detect as little as 10 pg of DNA/PCR mixture (Table 1).

We then determined the sensitivities of these primers for the detection of *G. duodenalis* (assemblage B) cyst DNA. DNA was extracted from stool specimens with four different concentrations of *G. duodenalis* cysts (i.e., 3,368, 674, 337, and 168 cysts/ml) by the FTA filter paper method. PCR amplifications were performed as described above. The most sensitive primer pair was defined as the one that could amplify DNA of *G. duodenalis* at the lowest numbers of cysts. The results demonstrated that the RH11/RH4 and GiarF/GiarR primer set was also the most sensitive primer set, which could detect as low as 168 cysts/ml (Table 1). The sensitivity of this primer set may be due to high copy numbers of the SSU rRNA gene in the organism. The presence of approximately 60 to 130 copies of rRNA gene per nucleus of *G. duodenalis*, arranged in tandem repeats, has been reported (1, 10). Moreover, the SSU rRNA sequence is more conserved than the other regions that were used as templates, which could be the explanation for why primers amplifying the SSU rRNA gene worked better.

The development of immunofluorescence assays has generally improved the sensitivity of detecting and quantitating fecal *Giardia* cysts and may allow accurate determination of prevalence rates and cyst excretion intensities compared to conventional microscopy (7). Although both immunofluorescence assay and PCR have been widely used, comparisons of sensitivities and specificities between these two procedures using human stool specimens have not been done. However, a study using calf stool specimens indicated that PCR was more sensitive than immunofluorescence assay (13).

Based on the above results, the most sensitive DNA extraction method (i.e., FTA filter paper) together with the most sensitive PCR method (i.e., that with the RH11/RH4 and GiarF/GiarR primer set) was compared to immunofluorescence assay in terms of sensitivities and specificities. Blinded evaluation of both techniques was performed using 70 stool samples (37 positive and 33 negative samples) which were collected from asymptomatic schoolchildren during a survey of parasitic infections in a primary school in Chachengsao Province, Thailand, in February 2006. *Giardia* cysts were microscopically identified using saturated sodium nitrate flotation. Stool specimens were stained with immunofluorescence antibody (Cellabs, United Kingdom) following the manufacturer's instructions and examined by fluorescence microscopy. Sensitivities and specificities were calculated using two-by-two tables and Epi Info version 6.04 software. A chi-square test was used to determine the significance between two proportions for sensitivities and specificities of the two diagnostic methods.

Using saturated sodium nitrate flotation as the gold standard, the sensitivities of the PCR method and the immunofluorescence assay were 97.3% (95% confidence interval [95% CI], 87.9 to 99.9%) and 91.9% (95% CI, 79.5 to 97.9%), respectively. The specificity of both PCR and immunofluorescence assay was 100% (95% CI, 91.3 to 100%). The difference between the sensitivities of both methods was 5.4% (95% CI, 0.9 to 16.7%). There were no significant difference between the sensitivities and specificities of the PCR and immunofluorescence assay ($P = 0.61$).

In conclusion, several PCR methods for the detection of *G. duodenalis* have been published; however, there was no standardized method. The present study has identified a highly sensitive PCR method for the detection of *G. duodenalis* by using FTA filter paper for DNA extraction together with the RH11/RH4-GiarF/GiarR primer set amplifying the SSU rRNA gene.

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Drinking Water: A Possible Source of *Blastocystis* spp. Subtype 1 Infection in Schoolchildren of a Rural Community in Central Thailand

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Abstract. In January 2005, a survey of intestinal parasitic infections was performed in a primary school, central Thailand. Of 675 stool samples, *Blastocystis* was identified with a prevalence of 18.9%. Genetic characterization of *Blastocystis* showed subtype 1 (77.9%) and subtype 2 (22.1%). Study of the water supply in this school was performed to find the possible sources of *Blastocystis*. *Blastocystis* from one water sample was identified as subtype 1, which had a nucleotide sequence of small subunit (SSU) ribosomal RNA (rRNA) gene that was 100% identical to that of *Blastocystis* infected in schoolchildren. Our information supports the evidence of water-borne transmission in this population.

INTRODUCTION

Blastocystis spp. is one of the most common intestinal protozoa reported in humans globally, with a high prevalence in developing countries.[1] In Thailand, epidemiologic studies of *Blastocystis* infection showed incidences as high as 10–40% in different populations.[2–4] Several forms of *Blastocystis* are observed in fecal specimens (i.e., amoeboid, vacuolar, avacuolar, granular, multivacuolar, and cyst). Of these, the vacuolar form is mostly recognized under microscopic examination. Transmission of *Blastocystis* occurs by the fecal–oral route. It has been postulated that thin-walled cysts are responsible for auto-infectivity in the host, whereas thick-walled cysts effect external transmission.[5] Transmission can be facilitated by the contamination of the environment, food, or water with excreted cysts from the reservoir hosts. *Blastocystis* cysts, which are 3–6 μm in diameter, remain viable under suitable conditions. Water-borne transmission of blastocystosis is indicated in a few studies.[3,6–8] Our previous epidemiologic study in army personnel showed a significant association of blastocystosis and drinking unboiled water.[3] It was postulated that infective cysts could possibly be obtained from contaminated drinking water.

To date, genotypic characterization using molecular techniques has been used to study the epidemiology of various infections including blastocystosis. These techniques are useful to identify routes of transmission and sources of infections. In this study, a cross-sectional study was conducted to investigate intestinal parasitic infections in schoolchildren of a primary school, Chacherngsao province, central Thailand, during January 2005. Genetic characterization of *Blastocystis* collected from schoolchildren using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis of the SSU ribosomal RNA (rRNA) gene was performed. Attempts to show the subtype of *Blastocystis* contaminated in the water supply provided at the school using PCR-RFLP and sequencing analysis of the SSU rRNA gene were also done to support evidence of water-borne transmission because a high prevalence of blastocystis was noticed. In addition, risk factors associated with blastocystosis in schoolchildren were also analyzed in this study.

MATERIALS AND METHODS

Study population. A cross-sectional study of intestinal parasitic infections was performed in a primary school that consisted of ~700 children between 6 and 13 years of age, Chacherngsao province, central Thailand, in January 2005. This study was approved by the Ethical Committee of the Royal Thai Army, Medical Department. Informed consents were obtained from parents or guardians before enrollment. A stool specimen from each enrolled student was examined for intestinal parasitic infections under light microscopy by wet preparation and formalin ethyl acetate concentration. Because wet preparation and formalin ethyl acetate concentration was less sensitive for the detection of *Blastocystis*, cultivation of *Blastocystis* using Jones medium supplemented with 10% horse serum was preformed as previously described.[9–11]

Questionnaires. To determine the risk factors and outcomes of parasitic infections, standardized questionnaires concerning demographic data, sanitary behaviors, source and treatment method of drinking water, animal contacts, and a history of gastrointestinal symptoms were included.

DNA extraction of *Blastocystis* in stool specimens. Positive samples of *Blastocystis* from culture medium were used for subtype identification. Extraction of genomic DNA of *Blastocystis* was performed using FTA filter paper as previously described.[12]

Water collection and extraction of DNA. To determine the possible source of *Blastocystis*, samples of the water supply within the school were collected and examined for *Blastocystis* using PCR. Approximately 3,000 mL of water was collected from five sources in the school area (i.e., water samples from three water reservoirs and two tanks of collected rainwater). DNA extraction was performed using a commercial kit, UltraClean Water DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA). Water samples were enumerated by vacuum filtering through 0.22-μm pore size, and DNA of any organisms present in water was extracted from the trapped filter, following the manufacturer's instructions. Extracted DNA was kept at –20°C until used for PCR amplification of the SSU rRNA gene of *Blastocystis*. In addition, PCR for detecting *Giardia duodenalis* using primers amplifying the glutamate dehydrogenase (*gdh*) gene was also performed.[13]

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RFLP analysis of the *Blastocystis* SSU rRNA gene. Genotypic characterization of *Blastocystis* was determined using PCR-RFLP analysis of 1,100 bp of the partial SSU rRNA gene. Extracted DNA was amplified by a pair of primers described by Clark.[14] Secondary PCR was performed using a specific pair of primers described by Bohm-Glöning and others.[15] The secondary PCR product produced the expected size of 1,100 bp. Digestion of PCR products was performed using three restriction enzymes, *Hinf*I, *Rsa*I, and *Alu*I endonucleases (Gibco BRL, Gaithersburg, MD), separated by 2% agarose gel electrophoresis and visualized under UV light and documented on high-density printing paper using a Uvisave gel documentation system I (Uvitech, Cambridge, UK).

Sequencing and phylogenetic analysis of the SSU rRNA gene of *Blastocystis*. To confirm the subtypes, nucleotide sequencing of the SSU rRNA gene of *Blastocystis* from schoolchildren and the water samples was conducted by Bioservice Unit, Bangkok, Thailand. Chromatograms were manually checked and edited using Sequencher version 4.0.5 (Gene Codes Inc., Ann Arbor, MI). Subsequently, nucleotide sequences of the SSU rRNA gene of *Blastocystis* obtained in this study were multiple aligned with a set of 33 other *Blastocystis* isolates retrieved from the GenBank database using BioEdit version 7.0.1. Phylogenetic analysis of SSU rRNA was carried out using MrBAYES version 3.1.2.[16] Bayesian analyses of the SSU rRNA dataset were performed using the GTR (general time reversible) + Γ (gamma distribution of rates with four rate categories) + I (proportion of invariant sites) model of sequence evolution, with base frequencies, the proportion of invariant sites, and the shape parameter α of the Γ distribution estimated from the data. In Bayesian analyses, starting trees were random, four simultaneous Markov chains were run for 500,000 generations, burn-in values were set at 30,000 generations, and trees were sampled every 100 generations. Bayesian posterior probabilities were calculated using a Markov chain Monte Carlo sampling approach implemented in MrBAYES version 3.1.2.[17] The analysis was carried out with the inclusion of the 16S-like rRNA gene from *Proteromonas lacerate* (U37108), an organism phylogenetically closely related to *Blastocystis* as the outgroup.[18]

Statistical analysis. The association between potential risk factors and *Blastocystis* infection was assessed by the χ^2 test with a 95% confidence interval (CI). Univariate analysis was performed using SPSS for Windows version 11.5 (SPSS, Chicago, IL). Odds ratios (ORs) with 95% CIs and *P* values were calculated to compare outcome among study groups. Logistic regression was performed for multivariate analysis to assess the independent association of risk factors and blastocystosis.

RESULTS

Prevalence of parasitic infections. Of 675 stool samples, 227 (33.6%) schoolchildren were positive for intestinal parasitic infections as shown in Table 1. Approximately 31% had a single parasitic infection, whereas 2.5% had mixed infections. Intestinal protozoa (i.e., *Blastocystis* [18.9%], *G. duodenalis* [5.6%], *Trichomonas hominis* [3.0%], and *Entamoeba coli* [2.5%]) were predominantly found in this population. Those who were infected with pathogenic parasites were treated with appropriate antiprotozoal or anthelminthic drugs.

Characterization of schoolchildren with *Blastocystis* infection. The characteristics of schoolchildren and the prevalence of *Blastocystis* infection are shown in Table 2. There was no significant difference among sex, age group, family income, parent's education, number of children per household, history of animal contacts (dogs or cats), type of drinking water at home (treated or untreated water), and gastrointestinal symptoms. However, the prevalence of *Blastocystis* infection in the children who were positive for *G. duodenalis*, *E. coli,* or *T. hominis* was 4.2 times greater than those who were not.

TABLE 1
Prevalence of intestinal parasitic infections in primary schoolchildren

Organism	Number infected	Percent infected
Blastocystis spp.	126	18.7
Giardia duodenalis	38	5.6
Opisthorchis viverrini	20	3.0
Entamoeba coli	18	2.7
Trichomonas hominis	17	2.5
Trichuris trichiura	7	1.0
Small intestinal fluke	7	1.0
Hookworm	5	0.7
Enterobius vermicuralis	3	0.4
Strongyloides stercolaris	2	0.3
Total	243	33.6

Genotypic characterization of *Blastocystis* in stools and water samples. Using a nested PCR amplification of the SSU rRNA gene of *Blastocystis*, ~ 54% (68/126) of samples were positive for PCR with the expected amplicon at 1,100 bp. Of 68 positive PCR samples, subtype characterization of *Blastocystis* was performed. The most common banding RFLP patterns of *Blastocystis* from schoolchildren were identical to subtype 1, with a prevalence of 77.9%. The remaining samples (22.1%) were positive for subtype 2. Figure 1 shows the representative *Blastocystis* RFLP banding patterns of subtype 1 and subtype 2 produced by *Hinf*I, *Rsa*I, and *Alu*I endonucleases, respectively. There was no mixed subtype infection detected in these PCR-positive samples. *Blastocystis* DNA was amplified from one water sample collected from a storage tank in the canteen (Figure 2). The other four water samples were negative for *Blastocystis* and *G. duodenalis* by specific PCR amplification.

Sequencing and phylogenetic analysis of the SSU rRNA gene of *Blastocystis*. From the schoolchildren's specimens, three and one samples of *Blastocystis* subtype 1 and subtype 2, respectively, were processed for nucleotide sequencing of the SSU rRNA genes. Nucleotide sequences of the SSU rRNA genes of *Blastocystis* subtype 2 were submitted to GenBank under accession numbers EF200010. The 1,700-bp sequences of *Blastocystis* subtype 1 from schoolchildren and water samples and 1,022-bp sequences of *Blastocystis* subtype 2 were multiple aligned with a set of 33 other *Blastocystis* isolates retrieved from the GenBank database. An inferred phylogenetic tree of SSU rRNA sequences of *Blastocystis* from the water samples and schoolchildren with 33 sequences of *Blastocystis* in the GenBank database and the outgroup, *Proteromonas lacertae*, was constructed to examine the genetic relationships. The rooted maximum-likelihood tree of *Blastocystis* sequence alignment identified nine clades of subtypes 1–9 with strong support by Bayesian posterior probabilities (BPs) of 100% for each clade (Figure 3). *Blastocystis* identified from the schoolchildren and the water sample were clustered with subtype 1 and showed 100% identity with our previously report *Blastocystis* (accession number AF439782).

TABLE 2
Characteristics of the enrolled primary schoolchildren and prevalence of blastocystosis

Characteristics	Number (%)	Prevalence of blastocystosis (%)	*P* value
Sex			
Male	336 (51.2)	65 (19.3)	
Female	320 (48.8)	57 (17.8)	0.614
Age group (years)			
< 9	235 (35.9)	42 (17.9)	
≥ 9	420 (64.1)	80 (19.1)	0.711
Parent's primary education			
Complete	82 (12.5)	19 (23.2)	
Incomplete	574 (87.5)	103 (17.9)	0.255
Number of children/household			
≤ 3	118 (18)	24 (20.3)	
> 3	538 (82)	98 (18.2)	0.591
Frequency of dog contact, three times or more/wk			
Yes	269 (41)	55 (20.4)	
No	387 (59)	67 (17.3)	0.310
Frequency of cat contact, three or more times/wk			
Yes	189 (28.8)	42 (22.2)	
No	467 (71.2)	80 (17.1)	0.129
Drinking water (at home)			
Untreated water	527 (80.3)	99 (18.8)	
Treated water (boiled or filtered)	129 (19.7)	23 (17.8)	0.802
Co-infection with other protozoan parasites			
Yes	29 (4.4)	20 (68.9)	
No	627 (95.6)	102 (16.3)	< 0.001
Total	656 (100)	122 (18.6)	

In addition, another subtype of *Blastocystis* isolate (accession number EF200010) identified from the schoolchildren formed a monophyletic group sharing within the clade of *Blastocystis* subtype 2 with a BP value of 100%.

Risk factors of *Blastocystis* infection. Using univariate and multivariate analysis, sex, age, family income, parent's education, number of children per household, history of animal contacts, and type and treatment method of drinking water at home were not significant risks of *Blastocystis* infection in these children. Those who had other intestinal protozoal infections, including *G. duodenalis*, *E. coli,* and *T. hominis,* had 11.3 times greater risk of getting *Blastocystis* infection (95% CI, 5.0–25.5; $P < 0.001$). When only *Blastocystis* subtype 1 was analyzed, this risk still remained, with an OR of 5.0 (95% CI, 2.1–12.0; $P = 0.001$). However, there was no significant association between this risk and *Blastocystis* subtype 2 (OR = 1.8; 95% CI, 0.2–14.6; $P = 0.448$).

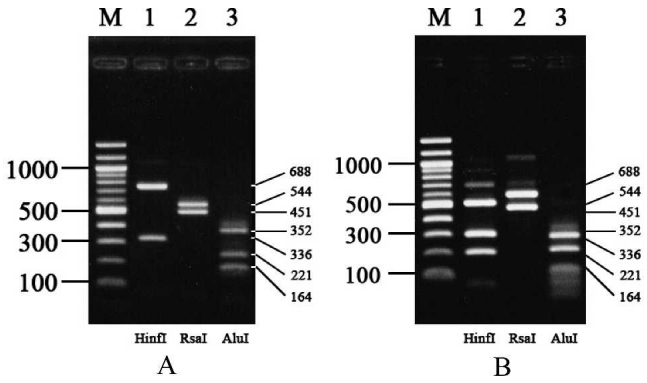

FIGURE 1. PCR-RFLP patterns of *Blastocystis* SSU rRNA gene from stool specimens of children. **A**, Subtype 1. **B**, Subtype 2. M, molecular marker (100-bp ladder). Lanes 1–3 show restriction enzyme profiles of 1,100 bp of the SSU rRNA gene digested by *Hinf*I, *Rsa*I, and *Alu*I, respectively.

DISCUSSION

Studies of *Blastocystis* infection in Thai children showed that the prevalence ranged from 0.8% to 45.2% depending on the study population and the detection methods.[19–22] The studies that used *in vitro* cultivation for the detection of *Blastocystis* infection showed rather high prevalences.[20,22] In this study, the highest prevalence of *Blastocystis* was found among intestinal parasitic infections. To understand the transmission of *Blastocystis* in this population, subtyping of *Blastocystis* from schoolchildren and water sources was performed. PCR-RFLP of the SSU rRNA gene has been widely used to characterize *Blastocystis,* which could differentiate the organism into clades, groups or subtypes, subgroups, ribodemes, and

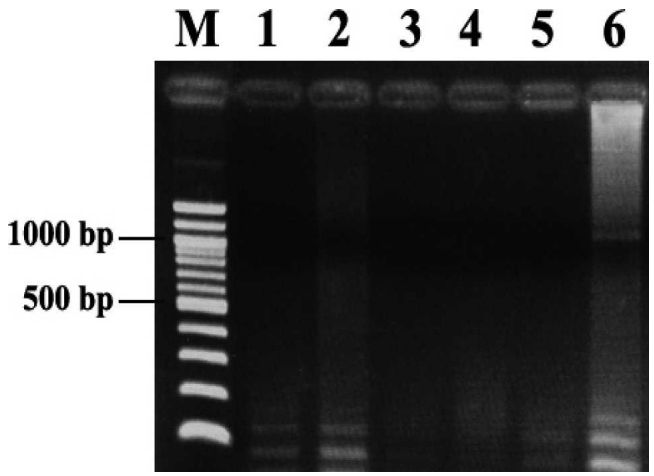

FIGURE 2. PCR product at 1,100 bp of the SSU rRNA gene of *Blastocystis* (Lane 6) detected in one water sample in a storage tank. M, molecular marker (100-bp ladder). Lanes 1–5 show negative amplification of other water samples.

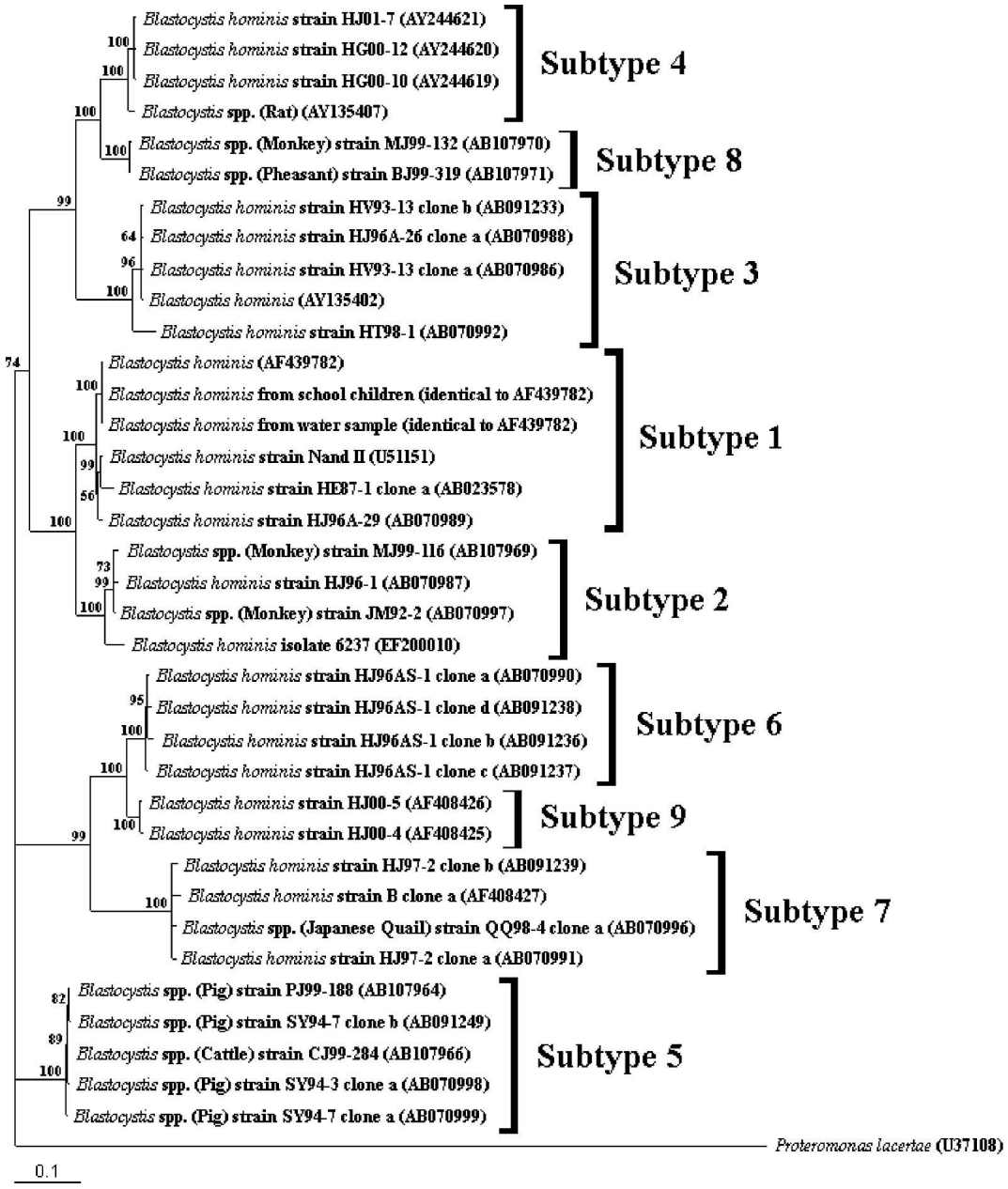

FIGURE 3. Maximum-likelihood phylogeny of *Blastocystis* isolates inferred from SSU rRNA gene sequences. The 1,700-bp sequences of *Blastocystis* subtype 1 from schoolchildren and water sample and 1,022-bp sequences of *Blastocystis* subtype 2 were used for the phylogenetic analysis.

clusters depending on the authors' criteria. However, because the *Blastocystis* SSU rRNA gene exhibits a high degree of genetic diversity, PCR-RFLP results may be very confusing or difficult to interpret. Thus, a consensus of *Blastocystis* subtypes has been recently provided based on the analysis of published SSU rRNA gene sequences.[23]

Distribution of *Blastocystis* subtypes is geographically different; most studies showed that subtype 3 was most frequently observed in humans (61%).[24] Our previous study showed that subtype 1 was the most predominant in Thailand, followed by subtypes 3 and 7.[25] A study in a different area of Thailand identified subtypes 5 and 6.[26] In this study, the most common subtype identified in the schoolchildren was subtype 1, followed by subtype 2. The absence of *Blastocystis* subtype 3 in this population might be simply explained by a different geographic distribution. Interestingly, *Blastocystis* subtype 3 is probably the only subtype of human origin. Recent studies conducted in urbanized cities such as Japan, Denmark, and Singapore where zoonotic transmission might be limited found that *Blastocystis* subtype 3 was predominant.[27–30] In contrast to a study from Jiangxi, China, subtype 1, previously identified in various kinds of animals, was predominantly found in humans.[31] This study was conducted in the rural community of central Thailand where zoonotic transmission of intestinal protozoal infection might occur.[32] On the other hand, the absence of *Blastocystis* subtype 3 could be caused by the limited sensitivity of the detection method for *Blastocystis* subtype 3.[28] Because nearly one half of the samples

gave a negative PCR result, we cannot rule out the existence of other subtypes. It has been suggested that the use of multiple primer pairs is very useful to make sure that all subtypes have been identified.[28]

Pathogenicity of *Blastocystis* is still not clear because of conflicting reports on clinical symptoms caused by *Blastocystis* infection. Most studies showed that most cases of blastocystosis were asymptomatic; however, symptomatic cases with non-specific gastrointestinal symptoms, including irritable bowel syndrome, were also reported.[33,34] It has been shown that the amoeboid form may be responsible for gastrointestinal symptoms because it presented predominantly in symptomatic patients.[35] Phylogenetic analysis of the SSU rRNA gene using arbitrarily primed PCR (AP-PCR) also showed distinct clades of *Blastocystis* between those of symptomatic and asymptomatic patients.[36] Thus, distinct subtypes of *Blastocystis* may have different role in its pathogenicity. In this study, no specific gastrointestinal symptoms were found in these *Blastocystis*-infected children.

Blastocystis has been detected in humans and wide varieties of animal hosts.[18,25,26,37] The evidence suggesting the possibility of blastocystosis as a zoonosis was from genotypic characterization of the organism in humans compared with those found in animals.[25,26] Interestingly, some subtypes can be identified in both humans and animals, which indicates a zoonotic potential of these subtypes. For example, subtype 1 could be identified both in humans and a very wide range of animals (i.e., pigs, horses, monkeys, cattle, rodents, chickens, quails, and pheasants).[25,37,38] In natural settings, subtype 5 was also identified both in humans and dogs from the same village in Thailand.[26] A recent study using animal models showed that several human subtypes of *Blastocystis* could infect chickens and/or rats, confirming the zoonotic potential of human *Blastocystis* isolates.[39] However, only a small number of epidemiologic studies have implicated zoonotic transmission of *Blastocystis*. Rajah and others[40] found that those who worked closely with animals had a higher prevalence of blastocystosis compared with those who did not. Additional evidence from epidemiologic studies is needed to support the role of zoonotic transmission.

In contrast, waterborne transmission of blastocystosis has been suggested by a number of epidemiologic studies. Our studies indicated that those who consumed untreated water had higher risk of getting *Blastocystis* infection.[3,6] Recently, it was identified that infections caused by subtype 3 were associated with drinking unboiled water.[7] Water-borne transmission of blastocystosis is not unexpected because *Blastocystis* cysts can survive in different types of water. A study by Suresh and others[8] showed that viable *Blastocystis* cysts were detected in sewage samples. In addition, it has been shown that cysts of *Blastocystis* could survive in chlorinated water at standard concentrations.[41] Drinking water used by schoolchildren in this school was mainly from rainwater that was stored in two tanks. No further treatment of drinking water by filtration or boiling was done. *Blastocystis* was identified by PCR but not conventional microscopy or *in vitro* cultivation. We detected *Blastocystis* subtype 1 in a water sample from one of these storage tanks. The nucleotide sequence of the SSU rRNA gene showed 100% identity to those of subtype 1 found in stool specimens of schoolchildren. Thus, it can be postulated that contaminated rainwater might be the source of *Blastocystis* infection in this school. Contamination of the rainwater could occur along the roof, pipe, or in the storage tank. Although we did not examine the possible sources of contaminants, it was likely that contaminations could be from droppings of some animals. As mentioned above, a wide range of animals could be a reservoir of *Blastocystis,* especially subtype 1. Unfortunately, we did not have the epidemiologic data to support the association of drinking water at school and blastocystosis because we only included the information of water at home and not at school in our questionnaire. In contrast, our epidemiologic data showed no significant association between the prevalence of *Blastocystis* infection with types of drinking water at home. As a result, *Blastocystis* infection was less likely to occur at home. Thus far, techniques to detect *Blastocystis* in water samples have not been standardized. The method used in this study was easy to perform and allowed using less amount of water samples (3,000 mL). Thus, this technique could be potentially applicable to detect *Blastocystis*. Further study for improving and validating the protocols for the detection and genotypic characterization of *Blastocystis* in water should be performed.

In this study, co-infections of *G. duodenalis* and *T. hominis* were significantly associated with *Blastocystis* infection, particularly subtype 1. It has been well recognized that water is the main route of transmission of *G. duodenalis*.[42] Although DNA of *G. duodenalis* could not be detected by PCR amplification in these water samples, this epidemiologic data possibly support evidence of water-borne transmission of these intestinal protozoa. This emphasizes the importance of providing safe drinking water for preventing these protozoa infections.

In conclusion, we describe the possibility of waterborne transmission of *Blastocystis* using molecular techniques. Subtype 1, which was shown in stool specimens of schoolchildren and drinking water in this school, supports the evidence of waterborne transmission of *Blastocystis*. This study draws attention to public health policy for development control program to reduce the morbidity of intestinal protozoa infections, especially waterborne zoonotic disease such as blastocystosis in schoolchildren.

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Multiple Modes of Transmission of Giardiasis in Primary Schoolchildren of a Rural Community, Thailand

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Abstract. In February 2005, we conducted a cross-sectional study to determine the prevalence and the risk factors of giardiasis in 531 primary schoolchildren of a rural community, Chacheongsao province, Thailand. Using both sedimentation and flotation techniques to detect *Giardia duodenalis*, the prevalence of giardiasis was 6.2%. Assemblage A, subgenotype II and assemblage B, subgenotype IV were identified by PCR-RFLP of glutamate dehydrogenase gene. Our data might indicate that, in this population, only assemblage A, subgenotype II of *G. duodenalis* was transmitted via water. Using multivariate analysis, significant risk factors for giardiasis were children of age 5–9 years, households with ≥ 3 children under the age of 12 years, low parental educational level, drinking bottled water, and living in close contact with dogs. Washing hands before meals had a protective effect. From these significant risk factors, multiple modes of transmission of *G. duodenalis* were suggested in this population.

INTRODUCTION

Giardiasis, a common intestinal protozoal infection caused by *Giardia duodenalis*, has gained attention as a neglected disease in both developed and developing countries.[1] Clinical presentations of giardiasis vary from asymptomatic carriage to acute and chronic diarrheal illness. Young children are at high risk of chronic infection resulting in nutritional malabsorption and failure to thrive.[2] Infection is acquired by the ingestion of viable cysts, which are transmitted through fecal–oral contamination. Waterborne outbreaks of giardiasis have been reported in developed countries including the U.S. and also countries in Europe, while the major risk factors in low-income countries were sanitation and personal hygiene.[3]

G. duodenalis isolated from humans and other mammals is morphologically identical. Molecular studies revealed a substantial level of genetic diversity between *G. duodenalis* isolates. *G. duodenalis* recovered from humans fall into the two major genetic assemblages, i.e., A and B. Other distinct groups, assemblages C, D, E, F, and G, are animal-specific.[3] However, assemblage A and B have also both been isolated from animals, indicating that these 2 assemblages have zoonotic potential. The existence of subgenotypes within each assemblage was also identified. Assemblage A comprises two distinct clusters, AI and AII, while assemblage B consists of BIII and BIV. To date, only one study showed strong evidence supporting zoonotic transmission of giaridiasis.[4]

Most studies in Thailand examined the prevalence of giardiasis especially in schoolchildren and in day-care centers.[5–7] However, at the present time, no information on risk factors of giardiasis in Thailand is available. In the present study, we conducted a cross-sectional study of giardiasis in rural primary schoolchildren to determine the prevalence and the associated risk factors. In addition, genotypic characterization of *G. duodenalis* isolates from these children was also performed. The epidemiology of the infection with different *Giardia* genotypes would significantly contribute to the understanding of its sources and modes of transmission.

MATERIALS AND METHODS

Study population. This cross-sectional study was undertaken in a rural community, Chacheongsao province, Central Thailand, in February 2005. The research protocol was approved by the Ethical Committee of the Medical Department, Royal Thai Army. This community comprised approximately 5,000 people. Most adults were farmers and laborers whose parents had migrated from northeastern Thailand. They have maintained their northeastern tradition and culture, such as dialect and dietary habits. We aimed at studying the prevalence and the risk factors for giardiasis in primary schoolchildren because of our survey in 2002 showing highest prevalence of giardiasis in this group. A large primary school located in the center of the community consisted of 793 students. A total of 531 stool specimens were collected from these children who voluntarily enrolled into the study with the informed consent of their parents.

Stool collection and examination. Stool specimens were examined for intestinal parasites immediately after the collection by wet-smear preparation in normal saline and Lugol's iodine solution. All specimens were then processed with the formalin/ethyl acetate sedimentation concentration and the sodium nitrate flotation techniques to detect *G. duodenalis* and other intestinal parasites. *Blastocystis hominis* was identified after a short-term in vitro cultivation using Jones' medium supplemented with 10% horse serum because this method is more sensitive than wet-smear preparation and concentration techniques.[8,9] The cultures were incubated at 37°C for 48–72 hours and then examined at 10× and 40× magnification under a light microscope. Every stool specimen was examined for *Cryptosporidium* spp. and microsporidia using modified acid-fast and Gram-chromotrope staining, respectively.

Genotypic characterization. The purified *Giardia* cysts were washed thrice with phosphate-buffered saline (PBS). DNA extraction was performed using FTA filter paper as previously described.[10] Genotypic characterization of *G. duodenalis* was determined by polymorphic sites using seminested PCR of a 432-bp region of the glutamate dehydroge-

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nase (*gdh*) gene and PCR-RFLP method described by Read et al. (2004).[11] Briefly, amplification of the *gdh* gene was performed using primer pairs of GDHeF/GDHiR and GDHiF/GDHiR. A total mixture of 50 μL contained DNA template using a piece of FTA filter paper, 1× PCR buffer, 1.0 U of *Taq* polymerase, 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 25 pmol of each primer. The PCR condition was as follows: 1 cycle of 94°C for 2 min, 56°C for 1 min and 72°C for 2 min, followed by 55 cycles of 94°C for 30 s, 56°C for 20 s and 72°C for 45 s and a final extension of 72°C for 7 min. RFLP analysis was performed by digesting 10 μL of the PCR product with 5 U of *Nla*IV in 1× enzyme buffer (New England Biolabs, Ipswich, MA) in a final volume of 20 μL for 3 h at 37°C. PCR products and restriction fragments were separated by electrophoresis in 2% agarose gel. Gels were stained with ethidium bromide and visualized under UV light and documented on high-density printing paper by using a UVIsave gel-documentation system (UVItech, Cambridge, England). DNA sequencing of PCR products was also performed to compare results with the sequences of the following GenBank entries: L40509 (*G. duodenalis* assemblage AI), L40510 (*G. duodenalis* assemblage AII), AF069059 (*G. duodenalis* assemblage BIII), and L40508 (*G. duodenalis* assemblage BIV). Multiple alignment and restriction map analysis were performed using BioEdit (version 7) software.

Questionnaires. To determine the risk factors and outcomes of giardiasis, standardized questionnaires for collection of demographic data, sanitary behaviors including cooking and eating habits, source and treatment method of drinking water, pets or animal contact, and also history of present gastrointestinal symptoms were used in this study. Diarrhea was defined as a change in their normal pattern of bowel movements and at least 3 loose stools during a 24-h period, and dysentery was defined as at least one passage of mucous bloody stool in 1 day. The weight and the height of each student were recorded at school to determine their nutritional status using Thailand's standard growth curve, Ministry of Public Health, Thailand, 1999. Parents of the enrolled students were asked to complete the questionnaires.

Statistical analysis. The association between potential risk factors and *G. duodenalis* carriage was assessed by the χ^2 test with a 95% confidence interval. Univariate analysis was performed using EpiInfo, version 6.04b. Odds ratios with 95% confidence intervals and *P* values were calculated to compare outcomes among study groups. Logistic regression using SPSS for Windows, version 9.6, was performed for multivariate analysis to assess the independent association of risk factors and *G. duodenalis.*

RESULTS

Of 793 students, 531 (67%) students were voluntarily enrolled into the study. The prevalences of parasitic infections in the study population are shown in Table 1. Protozoal infections were predominant in this group. *B. hominis* was the most common intestinal parasite found in this study (8.1%). *G. duodenalis* was the second most common, with a prevalence of 6.2%. Other common intestinal parasites reported in children, such as *Ascaris lumbricoides*, *Cryptosporidium* spp., and microsporidia, were not found. Those who were infected with pathogenic parasites were treated with appropriate antiparasitic drugs.

TABLE 1
Intestinal parasitic infections in 531 students

Intestinal parasitic infection	Number	Percent
Blastocystis hominis	43	8.1
Giardia duodenalis	33	6.2
Opisthorchis viverrini	21	3.9
Trichuris trichiura	8	1.5
Hookworm	7	1.3
Enterobius vermicularis	3	0.6
Strongyloides stercoralis	2	0.4
Other	17	3.2

Genotypic characterization. Of 33 samples, PCR amplification of the *gdh* gene was successful from 12 samples (42.4%). PCR-RFLP using *Nla*IV endonuclease and sequence analysis of the PCR products led to the identification of the *G. duodenalis* genotypes found in schoolchildren as assemblage A, subgenotype II in 41.7% (5/12) of the group and assemblage B, subgenotype IV in the other 58.3% (7/12) of the group.

Characteristics of the enrolled students. Table 2 shows the characteristics of the enrolled students. These schoolchildren ranged in age from 5 to 14 years old. Giardiasis was more prevalent in younger students. Prevalence in the students of age 5–9 years was 9.0%, which was significantly higher than those over 9 years old (2.9%) ($P = 0.004$, χ^2 test). The prevalences of giardiasis were not significantly different between groups consisting of differences in sex, nutritional status, and clinical symptoms (Table 2).

Risk factors of giardiasis. Table 3 shows univariate analysis of the risk factors for giardiasis in this population; it pointed at children ranging in age from 5 to 9 years old, households with ≥ 3 children under age 12 years, a low parental educational level (less than primary school), and people living in proximity to dogs for more than once a week. These groups had a greater risk of acquiring giardiasis. Multivariate analysis also confirmed that these risk factors were independently associated with giardiasis in this population (Table 4). Children

TABLE 2
Characteristics of enrolled students and prevalence of *Giardia duodenalis* infection

Characteristics	Total (%)	No. of *G. duodenalis* positive (%)	*P* value
Age group (years)			
≤ 7	103 (19.4)	9 (8.7)	
8	97 (18.3)	10 (10.3)	
9	90 (16.9)	7 (7.8)	
10	114 (21.5)	2 (1.7)	
11	64 (12.0)	3 (4.7)	
≥ 12	63 (11.9)	2 (3.2)	0.093
Sex			
Male	280 (52.7)	19 (6.8)	
Female	251 (47.3)	14 (5.6)	0.594
Parent occupation(s)			
Agricultural	486 (91.5)	32 (6.6)	
Other	45 (8.5)	1 (2.2)	0.345
Diarrhea			
No	449 (84.6)	29 (6.5)	
Yes	82 (15.4)	4 (4.9)	0.804
Nutritional status			
Normal	484 (91.1)	29 (6.0)	
Low normal and underweight	47 (8.9)	4 (8.5)	0.521
Total	531 (100)	33 (6.2)	

TABLE 3
Univariate analysis of risk factors of *Giardia duodenalis* infection

Characteristics	*Giardia duodenalis*		Crude odds ratio (95% CI)	*P* value
	Negative	Positive		
Age group (years)				
> 9	234 (97.1)	7 (2.9)	1	0.004
5–9	264 (91)	26 (9.0)	3.3 (1.4–7.7)	
Sex				
Female	234 (94.4)	14 (5.6)	1	0.565
Male	261 (93.2)	19 (6.8)	1.2 (0.6–2.5)	
No. of children of age < 12 years in home				
< 3	365 (95.3)	18 (4.7)	1	0.02
≥ 3	133 (89.9)	15 (10.1)	2.3 (1.1–4.7)	
Parent education				
At least primary school	435 (94.8)	24 (5.2)	1	
No education	63 (87.5)	9 (12.5)	2.6 (1.2–5.8)	0.017
Keeping dog(s) at home				
No	160 (96.4)	6 (3.6)	1	
Yes	338 (92.6)	27 (7.4)	2.1 (0.9–5.3)	0.094
Close contact to dog				
< 1/week	303 (95.6)	14 (4.4)	1	
≥ 1/week	195 (91.1)	19 (8.9)	2.1 (1.0–4.3)	0.037
Washing hands before meal				
Occasionally	348 (92.3)	29 (7.7)	1	
Every time	150 (97.4)	4 (2.6)	0.3 (0.1–0.9)	0.029
Drinking bottled water				
No	362 (95.0)	19 (5.0)	1	
Yes	136 (90.7)	14 (9.3)	1.9 (0.9–4.0)	0.062

whose parents had a lower educational level than primary school were at 2.4 times a greater risk of getting infected with *G. duodenalis*. Children of age 5–9 years old had a 1.3 times greater risk of getting infected. Having more than 3 children per household of an age under 12 years old increased by 2.5-fold the risk of contracting *Giardia*. Those who had history of contacting dogs more than once a week had a 2.3-fold greater risk for getting the infection. In addition, drinking bottled water was identified as the risk factor for giardiasis. Washing hands before every meal had protective effect against contracting *Giardia*.

Because only 12 samples were genotypically identified, we were unable to statistically determine the association between genotypes and risk factors. Considering these 12 characterized samples, those who consumed boiled or filtered water (4 of 12) had assemblage B, subgenotype IV of *G. duodenalis*. For those who did not consume boiled or filtered water, 5 and 3 children had assemblage A, subgenotype II and assemblage B, subgenotype IV of *G. duodenalis*, respectively. In addition, 2 of them consumed bottled water, which was identified as a risk for acquiring the infection, harbored assemblage A, subgenotype II of *G. duodenalis.*

TABLE 4
Multivariate analysis of risk factors of *Giardia duodenalis* infection*

Characteristics	Adjusted odds ratio	95% CI	*P* value
Age group (years)			
> 9	1		
5–9	1.3	1.0–1.6	0.045
Close contact to dog(s)			
< 1/week	1		
≥ 1/week	2.3	1.1–4.9	0.025
No. of children of age < 12 years in home			
< 3	1		
≥ 3	2.5	1.2–5.2	0.017
Washing hands before meal			
Occasionally	1		
Every time	0.3	0.09–0.8	0.022
Parent education			
At least primary school	1		
No education	2.4	1.0–5.6	0.041
Drinking bottled water			
No	1		
Yes	2.5	1.2–5.5	0.016

* Adjusted for sex, parent income, and parent occupation(s).

DISCUSSION

In this population, intestinal protozoal infections, i.e., blastocystosis and giardiasis, were predominant. These findings contrasted with most surveys done in Thailand, which showed a higher prevalence of helminthiasis among Thai schoolchildren.[5,12] The biannual mass chemotherapy for helminthic infection using albendazole in this population, as directed by the parasitic control program of the Ministry of Public Health, might account for our findings. This policy might also have affected the level of *G. duodenalis* infection in this population as well because albendazole can be used for the treatment of giardiasis.[13] The prevalence of giardiasis in Thai schoolchildren was previously reported in a few studies. In 1989, a survey in primary schoolchildren in Chiangmai, Northern Thailand, showed a prevalence of 7.7%,[14] while in 2002, a survey in Nan, another province in Northern Thailand, showed a prevalence of 5.5%.[5] However, the data in the present study cannot be compared with those previous studies because *G. duodenalis* was detected with different methods. Most studies only used wet preparations that might underdetect the prevalence of giardiasis. We used the flotation technique, a more sensitive method, for detection of *G. duodenalis*.

Most cases in this population were asymptomatic, so we

made the choice of conducting a cross-sectional rather than a case-control study. Because giardiasis is a chronic disease, some of the cases might not be recent infections. This would mean that persons who answered questions related to specific risks might have suffered from recall bias. However, most of the questions used in our study appeared to be general rather than specific in nature. Hence, the results should not be affected by this possibility. In several studies, outbreak of giardiasis especially in developed countries usually indicated waterborne transmission. Significant risks identified in these studies included drinking untreated water, swallowing water while swimming, and even drinking tap water.[15–20] In the present study, multivariate analysis showed that drinking bottled water presented a 2.5 times greater risk of acquiring giardiasis. However, most of the bottled water consumed by this population was distributed by regional water companies, and the quality of the bottled water has not been investigated in this study. The safety of the drinking water is ensured by filtration processes because cysts are relatively resistant to chlorine and ozone.[21]

In this study, we used PCR-RFLP of the *ghd* gene to characterize *G. duodenalis*. Although amplification of the *gdh* gene was less sensitive than other gene loci, such as SSU-rRNA gene, subgenotypes of *Giardia* could be differentiated using a single gene locus.[10,11] Only 42.2% of positive specimens (12 of 33) were successfully characterized for their genotype. From our findings, it might be postulated that, in this population, only assemblage A, subgenotype II of *G. duodenalis* was transmitted via water. However, there was no statistical measurement to support this finding because the sample size was too small. In addition, examination of water for *G. duodenalis* with genotypic characterization should be done.

Both assemblage A, subgenotype II and assemblage B, subgenotype IV of *G. duodenalis* can be identified both in humans and animals; hence, it is possible that these genotypes are zoonoses. However, epidemiological evidence supporting zoonotic transmission is rather limited. Until recently, molecular epidemiological evidence strongly supported giardiasis as a zoonosis. The study by Traub et al. (2004) showed that humans owning dogs infected with *Giardia* had a greater risk of getting parasitized than those who had not. In addition, some humans and dogs living in the same household harbored the same genotypes of *G. duodenalis*.[4] A recent study in Thailand showed that some dogs living in temple communities might serve as a source of *G. duodenalis* for human infection because infected human cases in this community contained assemblage A, which is similar to that found in dogs.[22] In the present study, we found that schoolchildren who had close contact with dogs had an approximately 2 times greater risk of acquiring giardiasis. In addition, assemblage A, subgenotype II and assemblage B, subgenotype IV were identified in these schoolchildren. Thus, both epidemiological data and *G. duodenalis* assemblages identified in these children might support the zoonotic transmission from dogs to human.

A study of clinical presentations of giardiasis in Dutch patients showed that assemblage A isolates were detected in patients with intermittent diarrhea, while assemblage B isolates were present in patients with persistent diarrhea.[24] Another case-control study showed the significant association between assemblage A, subgenotype AII infections and diarrhea.[25] In contrast, Cedillo-Rivera and colleagues (2003) reported that assemblages A and B exhibit no apparent differences in virulence, suggesting that host factors play a dominant role in determining the clinical course of the infection.[26] In this study, no specific symptoms were significantly observed in those infected children. In addition, all 12 children whose *Giardia* was characterized had no GI symptoms. Thus, further studies need to be carried out to confirm the correlation of the assemblages and their clinical symptoms.

Person-to-person transmission is well recognized in crowded populations with exposure to infected persons. In developed countries, several reports put the emphasis on this mode of transmission in day-care centers.[27] In developing countries, person-to-person transmission could occur in the community, especially in households with numerous children.[28] The prevalence of giardiasis in this population was significantly greater in younger children. Thus, it is not surprising that having $\geq$ 3 children under age 12 years and sharing the same house presented a 2.5 times greater risk of getting the infection. This indicates that person-to-person transmission is also an important transmission pathway of giardiasis in this community.

Personal hygiene is a critical factor for children with giardiasis worldwide. In this study, children who washed their hands before meals were at a lower risk for *Giardia* infection. Educational achievement, especially for those affected children, can only occur effectively through the support of a school health program.[22] To ensure that children are being adequately cared for at home, provision of health education to parents or caretakers needs to be addressed, particularly for parents who have had no formal education, as children under their care have a higher risk of getting infected with *Giardia*.

Most epidemiological studies of giardiasis indicated waterborne, foodborne, or person-to-person transmission, depending on the study population.[23] Multiple modes of transmission of giardiasis were previously reported in only a few studies, most of which were conducted in developed countries.[18,19,29,30] Two case-control studies were conducted in Auckland, New Zealand, in which infected cases included those of different regions, so it should not be surprising to identify multiple modes of transmission of giardiasis.[18,19] White et al. (1989) and Katz et al. (2006) also reported multiple modes of transmission of giardiasis in rather specific populations, i.e., residents, employees and children of nursing home and country-club residents, respectively.[29,30] Our study was conducted in a different setting, i.e., a rural community in Thailand, where the evidence of multiple modes of transmission of giardiasis, including person-to-person, zoonotic, and waterborne transmission, could be observed. This indicates that the nature of transmission of giardiasis varied depending on each population. Hence, control strategies of giardiasis in each population should be based on the epidemiological information for each population.

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Prevalence of Intestinal Parasitic Infections in Military Personnel and Military Dogs, Thailand

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Objective: *To determine the prevalence of intestinal parasitic infections and risk factors among military personnel and military dogs at the Military Dog Center, Veterinary and Remount Department, Royal Thai Army, Thailand.*

Material and Method: *A cross-sectional study was conducted in January 2006 to examine intestinal parasitic infections using wet preparation and, formalin-ethyl acetate concentration. Modified acid fast and gram-chromotrope stains were used to identify Cryptosporidium spp. and microsporidia, respectively. Culture for Blastocystis was performed using Jone's medium. Genotypic characterization of Blastocystis and Giardia duodenalis were also determined using PCR-RFLP. To determine the risk factors and outcomes of intestinal parasitic infections, standardized questionnaires were used in the present study.*

Results: *Of 317 military personnel, the prevalence of intestinal parasitic infections was 22.4%. Blastocystis was the most predominant intestinal protozoa infection of 14.5 % while G. duodenalis was only 1.3 %. The prevalence of other helminthic infections were 4.8% which were Strongyloides stercoralis (2.5%), Hookworm (1.0%), Opisthorchis viverrini (1.0%), and Taenia spp. (0.3%), respectively. Blastocystis subtype 1 was identified in 25 positive culture specimens while all 4 positive of G. duodenalis were analyzed as Assemblage B, subgenotype IV. The presented data could not indicate that intestinal parasitic infections and blastocystosis in this army population were significantly linked to risk association among groups with regard to rank, age group, working unit, area of residence, animal contact, source and treatment of drinking water. Of 189 military dogs, the prevalence of intestinal parasitic infections was only 3.7% which was Blastocystis sp. (2.6%), S. stercoralis (0.5%), and Entamoeba coli (0.5%), respectively.*

Conclusion: *The predominant intestinal parasites found in this population, such as Blastocystis sp. and G. duodenalis transmit to humans via fecal-oral route so that improvement of sanitation and personal hygiene should be emphasized.*

Keyword: *Intestinal parasitic infection, military personnel, military dog, Thailand*

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Parasitic infections in Thai military personnel still remain a public health concern in the army since the high prevalence of 53.9% and 55.7% were observed in the authors surveys in the military bases, central Thailand during 2000 and 2002, respectively[1,2]. The most prevalent protozoa found was *Blastocystis* infection followed by *Giardia duodenalis*. Others were soil-transmitted helminthes (hookworm, *Strongylodes stercoralis*) and food borne trematode (*Opisthorchis viverrini*). Privates had significantly higher risk of acquiring parasitic infections than noncommissioned officers and officers[2]. For effective control program of intestinal parasitic infection, base-line data including the prevalence and risk factors of parasitic infections in military personnel in other regions of Thailand are required. The authors aimed to investigate the prevalence of intestinal parasitic infections among military personnel and military dogs at the Military

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Dog Center, Veterinary and Remount Department, Nakornrachsrima province, the frontier of the north-east region of Thailand. Since some of these parasites can transmit to humans from animals, the role of zoonotic transmission in this military base was determined. Associated risk factors were also analyzed using standardized questionnaires.

Material and Method

Study population

A cross-sectional study of intestinal parasitic infections was undertaken in January 2006 among military personnel and military dogs at the Military Dog Center, Veterinary and Remount Department, Royal Thai Army, Nakornrachasrima province, Thailand. Research protocol was approved by the Ethical Committee of Medical Department, the Royal Thai Army (no.138/2005). A total of 317 stool specimens were collected from military personnel including privates, noncommissioned officers, officers, and other employees, who voluntarily enrolled into the present study with the informed consent. Additionally, 189 stool specimens were also collected from dogs, trained for military missions at the Military Dog Center. These dogs were composed of German Shepherd, Labrador Retriever, Rottweiler, Belgian Malinois, Doberman, Dalmatian and others.

Stool collection and examination

Stool specimens were examined for intestinal parasites after the collection by wet smear preparation in normal saline and Lugol's iodine solution. All specimens were then processed for formalin/ethyl-acetate concentration. For genetic characterization of *Giardia*, sodium nitrate floatation was performed to collect *Giardia* cysts. For detecting *Blastocystis*, a short-term *in vitro* cultivation was performed for each stool sample using Jone's medium supplemented with 5-10% horse serum[3,4]. The cultures were incubated at 37°C for 48-72 hours and then examined at objective 10X and 40X under a light microscope. The composition of Jone's medium previously described[3] was prepared as follows; dissolve 1.244 g of Na_2HPO_4 in 131.25 mL distilled water, 0.397 g of KH_2PO_4 in 43.75 mL distilled water, and 7.087 g of NaCl in 787.50 mL distilled water, respectively. The three solutions were mixed together to a final volume of 962.5 mL, then discard 12.5 mL of the mixture. Add 1 g of yeast extract (Oxoid) into the mixture. Autoclave the solution and leave until it is cool. Add 5-10 mL of horse serum to 95-90 ml of the sterile medium. Aliquot 3-5 ml of the medium into a sterile screw cap tube, kept at 4°C until used. The present study also examined for oocysts of *Cryptosporidium* spp. and spores of microsporidia using modified acid-fast and gram-chromotrope stains, respectively.

Genotypic characterization

The purified *Giardia* cysts were washed thrice by phosphate buffered saline (PBS). DNA extraction was performed using FTA filter paper as previously described[5]. Genotypic characterization of *G. duodenalis* was determined by polymorphic sites using semi-nested PCR of a 432 bp region of the glutamate dehydrogenase (*gdh*) gene and PCR-RFLP method described by Read et al[6]. Briefly, amplification of *gdh* gene was performed using primer pairs of GDHeF/GDHiR and GDHiF/GDHiR. A total mixture of 50 ml contained DNA template using a piece of FTA filter paper, 1x PCR buffer, 1.0 U of *taq* polymerase, 1.5 mM $MgCl_2$, 0.2 mM dNTP and 25 pmol of each primer. The PCR condition was as follows; 1 cycle of 94°C for 2 min, 56°C for 1 min and 72°C for 2 min, followed by 55 cycles of 94°C for 30 s, 56°C for 20 s and 72°C for 45 s and a final extension of 72°C for 7 min. RFLP analysis was performed by digesting 10 ml of the PCR product with 5 U of *Nla*IV in 1X enzyme buffer (New England Biolabs, England) in a final volume of 20 ml for 3 h at 37°C. PCR products and restriction fragments were separated by electrophoresis in 2 % agarose gel, respectively. Gels were stained with ethidium bromide and visualized under UV light and documented on high-density printing paper by using a UV-save gel documentation system I (UVItec, Cambridge, United Kingdom). DNA sequencing of PCR products were also performed to confirm with the sequence of GenBank accession number; L40509 (*G. duodenalis* assemblage AI), L40510 (*G. duodenalis* assemblage AII), AF069059 (*G. duodenalis* assemblage BIII), and L40508 (*G. duodenalis* assemblage BIV). Multiple alignment and restriction map analysis were performed using program Bioedit version 7.

Positive samples of *Blastocystis* from culture medium were used for subtype identification. Genomic DNA of *Blastocystis* was extracted using FTA filter paper as previously described[5]. A pair of primers described by Clark et al[7] , was used as a primary primer while secondary PCR was performed using a specific pair of primers described by Bohm-Gloning et al[8]. The secondary PCR product produced the expected size of 1100-bp. Genotypic characterization of *Blastocystis* was determined using PCR-RFLP analysis of 1100 bp of partial SSU-rRNA gene. Digestion of PCR products

was performed using three restriction enzymes, *Hinf*I, *Rsa*I, and *Alu*I endonucleases (Gibco, BRL, Gaithersburg, Md.), separated by 2% agarose gel electrophoresis and then visualized under UV light and documented on high density printing paper using a UVsave gel documentation system I (UVItec, Cambridge, United Kingdom). To confirm the subtypes, nucleotide sequencing of the SSU rRNA gene of *Blastocystis* was conducted by Bioservice Unit, Bangkok, Thailand. Chromatograms were manually checked and edited using Sequencher version 4.0.5.

Questionnaires

To determine the risk factors and outcomes of intestinal parasitic infections, standardized questionnaires concerning demographic data**,** sanitary behaviors including cooking and eating habits, source and treatment method of drinking water, pets or animal contact and also history of present gastrointestinal symptoms were used in the present study. All participants were asked to complete the questionnaires when they provided their specimens. Diarrhea was defined as a change in their normal pattern of bowel movements and at least 3 loose stools during a 24-hour period. Dysentery was defined as at least one passage of mucous-bloody stool in 1 day.

Statistical analysis

The association between potential risk factors and intestinal parasitic infections was assessed by the chi-square test with a 95% confidence interval using EpiInfo version 6.04b. Univariate analysis was performed using SPSS for Windows version 11.5 (SPSS, Chicago, IL). Odds ratios with 95% confidence intervals and p-values were calculated to compare the outcome among the study groups. Logistic regression was performed for multivariate analysis to assess the independent association of risk factors and blastocystosis.

Results

Of 317 enrolled studied population, 182 (57.4%) were military personnel, including privates (18, 9.9%), non-commissioned officers (141, 82.4%), and officers (23, 12.6%). Others were civilian personnel (135, 42.6%) who had been hired to work as dog caretakers (49, 36.3%) and service workers (86, 63.7%) at this army base. Approximately 58.4% originated from the North-eastern region, 22.1% from the central part of Thailand, 5.4% from the North and, 14.1% from other regions. Other characteristics of the studied population are shown in Table 1. Seventy-two (22.4%) were found positive for intestinal parasitic infections. As shown in Table 2, *Blastocystis* was the most common protozoa found in this population with the prevalence of 14.5%, followed with *S. stercoralis* (8, 2.5%), *E. coli* (7, 2.2%), *G. duodenalis* (4, 1.0%), hookworm (3, 1.0%), *O. viverrini* (3, 1.0%), *Taenia* spp. (1, 0.3%), *Endolimax nana* (2, 0.6%). *Cryptosporidium* spp. and microsporidia were not found. Those who were

Table 1. Demographic data of military personnel at the Military Dog Center, Veterinary and Remount Department, Royal Thai Army, Thailand

Characteristic	Number (%)	No. infected (%)	p-value
Rank			
Private	18 (5.7)	2 (11.1)	
Commisioned ofiicer	23 (7.3)	5 (21.7)	
Noncommissioned officer	141 (44.4)	33 (23.4)	
Civilian employee	135 (42.6)	31 (23.0)	
Total	317 (100)	71(22.4)	0.698
Gender			
Male	292 (92.1)	66 (22.6)	
Female	25 (7.9)	5 (20.0)	
Total	317 (100)	71(22.4)	0.842
Unit			
Medical corps	8 (2.8)	1 (12.5)	
Engineer corps	28 (9.9)	6 (21.4)	
Veterinary and dog	109 (38.4)	25 (22.9)	
Office and others	139 (48.9)	33 (23.7)	
Total	284 (100)	65 (22.9)	0.901
Level of education			
Primary school	79 (24.9)	23(29.1)	
Secondary school	138 (43.5)	30 (21.7)	
Diploma or equal	72 (22.7)	15 (20.8)	
Bachelor degree or higher	23 (7.3)	3 (13.0)	
Total	312 (100)	71(22.8)	0.350
Age (years)			
< 30	19 (6.2)	1 (5.3)	
31-40	47 (14.8)	10 (21.3)	
41-50	108 (34.1)	28 (25.9)	
51-60	133 (42)	31 (23.3)	
Total	307 (100)	70 (22.8)	0.404
Drinking water			
Boiled	144 (45.4)	35 (24.3)	
Not boiled	173 (54.6)	36 (20.8)	
Total	317 (100)	71 (22.4)	0.457
Current residence			
In the camp	236 (76.6)	51 (21.6)	
Outside the camp	72 (22.4)	18 (25.0)	
Total	308 (100)	69 (22.4)	0.546

Table 2. Intestinal parasitic infections among 317 military personnel and 189 military dogs at the Military Dog Center, Veterinary and Remount Department, Royal Thai Army, Thailand

Intestinal parasite	Positive	Percent
Military personnel (n = 317)		
Blastocystis sp.	46	14.5
Strongyloides stercoralis	8	2.5
Giardia duodenalis	4	1.3
Hookworm	3	1.0
Opisthorchis viverrini	3	1.0
Taenia spp.	1	0.3
Entamoeba coli	7	2.2
Endolimax nana	2	0.6
Total	74	22.4
Military dogs (n = 189)		
Blastocystis sp.	5	2.6
Strongyloides stercoralis	1	0.5
Entamoeba coli	1	0.5
Total	7	3.7

infected with pathogenic parasitic infection were treated with proper antiparasitic drugs. In addition, instruction to prevent themselves from acquiring intestinal parasitic infections i.e. foodborne parasite, waterborne parasite and soil-transmitted helminthes were provided to the studied population.

The prevalence of intestinal parasitic infections was not significantly different among groups regarding gender, rank, age group, working unit, area of residence, animal contact, source and treatment of drinking water. Using univariate and multivariate analysis, no significant risk factors were identified regarding gender, rank, age group, working unit, area of residence, animal contact, source and treatment of drinking water. Of 46 positive culture of *Blastocystis* sp., only 25 were available for genetic characterization and were identified as subtype 1. In addition, using PCR-RFLP of the *gdh* gene, all 4 positive human cases of *G. duodenalis* were genetic and characterized as assemblage B, subgroup IV.

All dogs enrolled in the present study were 1-7 years old and had their individual dog caretaker. Of 189 dogs, 7 (3.7%) were positive for intestinal parasitic infections which were *Blastocystis* (5, 2.6%), *S. stercoralis* (1, 0.5%), *and E. coli* (1, 0.5%), respectively. The dog, infected with *S. stercoralis*, was treated with the anti-parasitic drug while others had non-pathogenic protozoa infection and required no treatment.

Genotypic characterization of 5 positive cultures of *Blastocystis* sp. found in dogs was unsuccessfully identified for subtype due to specimen storage.

Discussion

The populations enrolled into the present study originated from all regions of Thailand, approximately 60% of them were from the Northeast. During the present study, privates were on active duty outside the military base, thus only 18 (5.7%) privates were enrolled into the present study. In the present study, the prevalence of intestinal parasitic infections in military personnel was 22.4%. The authors' previous study demonstrated a significantly increased risk of acquiring intestinal parasitic infections among privates aged 21-23 years old, especially those who finished less than secondary school[2]. Thus, the prevalence of intestinal parasitic infections found in this population could have been higher if there were more enrollment of the private group. In this population, parasite control program using bi-annual mass chemotherapy of mebendazole for military personnel had been administered which could somehow control the prevalence rate of intestinal helminthic infections to be as low as 4.7%. However, effective prevention and control of soil-transmitted helminthes i.e. *S. stercoralis* and hookworm infection is still needed in this population. Compared to other studies conducted in the Northeast region[9], 1.3% of other infections were *O. viverrini* and *Taenia* spp., which were less prevalent than expected. Thus, a small number of privates who enrolled into the present study could account for the low prevalence of *O. viverrini* and *Taenia* spp.

In Thailand, the prevalence of blastocystosis, an intestinal protozoal infection, extend over a wide range which were approximately 0.8-45% depending on the methods of detection and studied populations. A significance of blastocystosis has increasingly gained more attention since the high prevalences have been reported in all age groups. In the present study, blastocystosis, detected by *in vitro* cultivation using Jone's medium, a more sensitive method compared to formalin-ethyl acetate sedimentation technique, was predominant among military personnel and dogs at the military dog center. This finding agreed with a few studies of intestinal parasitic surveys performed at other army bases in Thailand, which also showed the highest prevalence of blastocystosis among Thai military personnel i.e. 21.9%, 44.4% and 36.9% in the central region of Thailand[1, 2, 10]. Interestingly, the present

result was similar to that reported from the study in Hondurus which reflected a common *Blastocystis* infection. U.S. military both servicemen and women who were assigned to do their duty in Hondurus acquired blastocystosis at the highest prevalence of 35.8%[11]. The high prevalences of blastocystosis was also demonstrated in children i.e. 45.2% in an orphanage, Pathumthani province[12] and 8.1% in a primary school, central Thailand[13]. In contrast to studies conducted in other groups using formalin-ethyl acetate sedimentation, a less sensitive technique, showed rather lower prevalence of blastocystosis i.e. 0.19% in primary school children in central Thailand[14], 0.8% in school children in Northern Thailand, 4.1% in Thai laborers[9] and 6.1% in school children, in Central Thailand[15].

Blastocystis sp. has been identified both in humans and a very wide range of animals. Recent studies confirmed zoonotic transmission of blastocystosis[16]. Cyst of *Blastocystis* served as the infective stage and transmits via fecal-oral route. Like other protozoa, transmission of blastocystosis can occur through waterborne or food borne. Thus, source of infection of *Blastocystis* could have come either from human or animal excreta, which contaminated into water or food. The presented data could not indicate that blastocystosis in this army population was significantly linked to any risk association among groups with regard to rank, age, working unit, area of residence, animal contact, source and treatment of drinking water.

Genetic characterization of *Blastocystis* revealed several subtypes including subtypes 1 to 9[17]. In the present study, genetic characterization of *Blastocystis* revealed only subtype 1 while other subtypes could not be excluded for the existence since only 25 samples (54.3%) were available for genetic characterization. These military personnel who harbored *Blastocystis* subtype 1 did not show any clinical symptoms. *Blastocystis* subtype 1 has been identified both in humans and a very wide range of animals i.e. pigs, horses, monkeys, cattle, rodents, chickens, quails and, pheasants[18,19]. The authors' previous study of *Blastocystis* isolated from dogs and humans living in a localized endemic community in Thailand provided molecular-based evidence supporting the zoonotic potential of *Blastocystis* in dogs[16]. Unfortunately, the authors did not compare the subtype of *Blastocystis* isolated from military personnel and military dogs since the subtype of *Blastocystis* from dogs could not be determined. Studies of blastocystosis in military personnel have been conducted in Thailand. It was shown that consuming neither filtered nor boiled water was independently associated with blastocystosis[1]. Another study also showed that privates who had education lower than the secondary school level had a significantly greater risk of *Blastocystis* carriage[2].

Giardiasis, the most commonly notified waterborne disease and causes a wide range of clinical symptoms, has been reported worldwide. The prevalence of giardiasis tends to be more common in young children than adults. In Thailand, the prevalence studies showed 37.7 % in orphans[12] while 1.4-7.7% among school children[10,13,14,20-22]. The prevalence of giardiasis in Thai military personnel was previously reported with the prevalence of 3.7%[2]. There are two major genetic assemblages of *G. duodenalis* found in humans i.e. assemblages A and B. It has been shown that assemblage A and B were isolated from animals, thus these 2 assemblages have a zoonotic potential. Other assemblages C, D, E, F and G, are found only in animals[23]. Subgenotypes within each assemblage were also identified. Assemblage A comprises of subgenotypes AI and AII while assemblage B consists of subgenotypes BIII and BIV. A study showed evidence supporting zoonotic transmission of giardiasis[24]. In the present study, the authors used PCR-RFLP of *ghd* gene to study both assemblage and subgenotype of *G. duodenalis*. All 4 positive specimens were successfully characterized as Assemblage B, subgenotype IV. It is possible that the infective stage could have come from the same source. However, there was no statistical measurement to support any risk association due to the small number of sample size. This preliminary data of *G. duodenalis* subgenotype also urge us to investigate their source of transmission in other population.

The present study showed the low prevalence of intestinal parasitic infections in military dogs. The parasite control program using bi-annual mass chemotherapy for military dogs accounted for the findings. In addition, dog health care and well-being had been routinely handled by the veterinarian. Safe food and clean water were regularly inspected and provided to all dogs. As a result, only one military dog was infected with soil-transmitted helminth, *S. stercoralis*. The infection was acquired principally by skin penetration of infective filariform larvae of *S. stercoralis* from the soil or playground. The other 6 dogs were infected with non-pathogenic protozoa which included *Blastocystis* sp. and *E. coli*. The morphology of *Blastocystis* sp. obtained from dogs when observed in Jone's medium was similar to those

found in humans. Since transmission route of *Blastocystis* sp. to humans was demonstrated by the presence of zoonotic strains[25], genetic characterization of *Blastocystis* found in both humans and dogs could help understanding the epidemiology of the organism. Unfortunately, genotype of *Blastocystis* detected in military dogs could not be performed due to long-term specimen storage.

In conclusion, the present study demonstrated that parasitic infection acquired via fecal-oral route is still a significant problem among military personnel. Base-line information of parasitic infection among military personnel in other regions of Thailand need further studies since continuous surveillance programs for enteric parasites are warranted to reduce morbidity. Moreover, health education provided to the affected population could enable them to protect themselves against infection.

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ความชุกของโรคติดเชื้อปรสิตในลำไส้ในทหารและสุนัขทหารในประเทศไทย

เสาวนีย์ ลีละยูวะ, สุรเดช ศิริพัฒนพิพงษ์, ทวี นาคหล่อ, ปานจิต ธรรมศรี, มฑิรุทธ มุ่งถิ่น

วัตถุประสงค์*: ศึกษาหาความชุกและปัจจัยเสี่ยงของโรคติดเชื้อปรสิตในลำไส้ในทหารและสุนัขทหารที่ศูนย์สุนัขทหาร กรมการสัตว์ทหารบก ประเทศไทย*

วัสดุและวิธีการ*: การศึกษาชนิดตัดขวาง โดยดำเนินการเก็บอุจจาระจากกำลังพลทหาร และสุนัขทหาร ในเดือน มกราคม พ.ศ. 2549 และตรวจโดยใช้ทั้งการตรวจธรรมดาและวิธีเข้มข้นชนิด formalin-ether ย้อมสี modified acid fast และ gram-chromotrope เพื่อตรวจหา Cryptosporidium และMicrosporidia ตามลำดับ เพาะเชื้อเพื่อตรวจหา Blastocystis ใน Jone's medium ศึกษา genotype ของ Blastocystis and Giardia duodenalis โดยใช้ PCR-RFLP รวมทั้งหาปัจจัยเสี่ยงของการติดโรคพยาธิในลำไส้โดยการใช้แบบสอบถามมาตรฐาน*

ผลการศึกษา*: จากอุจจาระที่เก็บได้ทั้งหมด 317 ตัวอย่างจากทหาร พบความชุกของโรคติดเชื้อปรสิตในลำไส้ 22.4% โดยโปรโตซัวมากที่สุดพบคือ Blastocystis 14.5% และรองลงมาคือ G. duodenalis 1.3% ความชุกของหนอนพยาธิทั้งหมดมี 4.7% โดยพบ Strongyloides stercoralis (2.5%), Hookworm (1.0%), Opisthorchis viverrini (1.0%), และ Taenia spp. (0.3%), ผลการทำ PCR-RFLP พบ Blastocystis subtype 1 จำนวน 25 ราย G. duodenalis Assemblage B, subgenotype IV จำนวน 4 ราย นอกจากนั้นการตรวจอุจจาระสุนัขทหาร 189 ตัว พบความชุกของโรคติดเชื้อปรสิตในลำไส้ 3.7% โดยพบ Blastocystis มากที่สุด (2.6%) ตามด้วย S. stercoralis (0.5%) และ Entamoeba coli (0.5%) จากการวิเคราะห์ข้อมูลทางสถิติ ไม่พบปัจจัยเสี่ยงของโรคติดเชื้อปรสิตในลำไส้ในทหารที่สัมพันธ์กับ ยศ กลุ่มอายุ หน่วยที่สังกัด ภูมิลำเนา น้ำดื่ม ประวัติสัมผัสสัตว์*

สรุป*: การสำรวจโรคติดเชื้อปรสิตในลำไส้ในทหารและสุนัขทหารในประเทศไทย พบความชุกของโปรโตซัวที่ติดต่อทาง fecal-oral คือ Blastocystis sp. และ G. duodenalis ตามลำดับ การป้องกันควรเน้นสุขอนามัยส่วนบุคคล*