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Prevalence of giardiasis and genotypic characterization of *Giardia duodenalis* in hilltribe children, Northern Thailand

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Abstract. A cross-sectional study was performed to determine the prevalence of giardiasis in hilltribe children of 2 different remote districts (Mae-chaem and Hod), Chiang Mai, Northern Thailand from November 2006-April 2007. The overall prevalence of giardiasis was 5.2%. Genetic characterization of *Giardia duodenalis* isolated from these children was performed using PCR methods specific for small subunit ribosomal rRNA (SSU-rRNA) and glutamate dehydrogenase (*gdh*) gene. This study shows that the distribution of *Giardia* assemblages varied in these 2 populations. Assemblage BIV was found predominantly in children from Hod District while assemblage AII was more common in children from Mae-Chaem District. Our result showed that assemblage A was significantly associated with loose/watery stool (p= 0.001). In addition, children harbouring assemblage B had shed a significantly higher number of cysts (p= 0.019) in stools than those infected with assemblage A. Further study on the epidemiology of giardiasis especially risk factors associated with genotyping would help to understand the nature of this disease in each population.

INTRODUCTION

Giardiasis, caused by *Giardia duodenalis*, is one of the most common intestinal protozoal infections reported worldwide. It has been estimated that there are 280 million symptomatic cases of giardiasis each year (Lane & Lloyd, 2002). One of the high risk groups are children particularly those in daycare settings, orphanages and primary schools (Polis *et al*., 1986; Saksirisampant *et al*., 2003; Ratanapo *et al*., 2008). In Thailand, the prevalence of giardiasis in children varies from 1.3 to 37.7% (Wongstitwilairoong *et al*., 2007). Clinical manifestations of giardiasis can range from asymptomatic cyst passage to acute or chronic diarrhoea. Severe consequence of giardiasis such as malabsorption and failure to thrive could be found in young children (Wolfe, 1992).

Different clinical outcomes of giardiasis could be attributed to host factors such as immunity and nutritional status and also pathogen factors. Recently, a few studies determined the correlation between clinical outcomes of giardiasis and *G. duodenalis* assemblages (Homan & Mank, 2001; Read *et al*., 2002; Cedillo-Rivera *et al*., 2003; Haque *et al*., 2005; Sahagún *et al*., 2008; Abdel-Moneim & Sultan, 2008; Kohli *et al*., 2008; Peréz Cordón *et al*., 2008; Mohammed Mahdy *et al*., 2009). At least 8 assemblages (A-H) of *G. duodenalis* have been reported (Feng & Xiao, 2011). Assemblages A and B are found primarily in humans and occasionally in animals while assemblages C, D, E, F and G

are animal specific (Mayrhofer *et al*., 1995; Monis *et al*., 1996; 1999; 2003). Assemblage A has two distinct clusters, AI and AII while assemblage B consists of BIII and BIV (Monis *et al*., 1996). So far, the association between clinical outcomes and *G. duodenalis* assemblages is inconclusive. A few studies have shown that those infected with assemblage A were more likely to have symptoms (Haque *et al*., 2005; Peréz Cordón *et al*., 2008; Sahagún *et al*., 2008). In contrast, Mohammed Mahdy *et al.* (2009) found that assemblage B infection was significantly correlated with clinical symptoms of giardiasis. A study in Dutch patients showed a strong correlation between assemblage A and intermittent diarrhoea, while assemblage B was present in patients with persistent diarrhoea (Homan & Mank, 2001). However, other studies could not find this association (Cedillo-Rivera *et al*., 2003; Abdel-Moneim & Sultan, 2008; Kohli *et al*., 2008).

In Thailand, a few ethnic minority groups (hill tribes) live in mountainous areas mostly along the Thai-Myanmar border. One of the largest hill tribes is the Karen. Children living in these remote areas may have a higher risk of parasitic infections due to poor sanitary conditions, socio-economic status and hygienic habits. A recent study in Sangkhlaburi, Western Thailand showed that giardiasis was predominant in Karen children (Wongstitwilairoong *et al*., 2007). Most studies of giardiasis in Thailand were conducted only in some particular groups such as school children and orphans. In addition, information of the distribution of *G. duodenalis* assemblages is also limited. Therefore, the present study aimed to determine the prevalence and the genotype of *G. duodenalis* in hilltribe children living in 2 districts of Chiangmai Province, Northern Thailand.

MATERIALS AND METHODS

Study Area and Study Population

This cross-sectional school-based study was conducted between November 2006 and April 2007. The research protocol was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University. This survey of *G. duodenalis* infection was performed among hilltribe children of 2 mountainous areas, Mae-Chaem and Hod Districts, Chiang Mai Province, Northern Thailand, who attended community schools in each area. These schools offered day care for younger children and education for the older ones. Mae-Chaem District is about 150 km west of Chiang Mai whereas Hod District is about 89 km south of Chiang Mai. Enrolled children were from 5 tribes which were Karen, Mong, Lava, Lesor and other. Stool specimens were collected from these children, who voluntarily enrolled into the study, with the written informed consent from their parents. Personal data of each child was provided by their parents.

Stool Examination

A single stool sample was collected from each child. All stool specimens were kept at 4ºC with no preservation until examined at the Department of Parasitology, Faculty of Medicine, Chulalongkorn University. The stool consistency (form, soft, loose, and watery) of each fecal sample was recorded by one laboratory technician throughout the study. All fecal specimens were examined for *G. duodenalis* using simple wet preparation and formalin-ethyl acetate sedimentation under light microscope. Approximately 2 g of each fecal sample was used for the sedimentation technique. Using pellets from the sedimentation, intensity of *Giardia* cyst was scored as the average number of ten high power field counts. The grading was determined as described by Almeida *et al.* (2006), i.e., 1-2 cysts = + (very low), 3-10 cysts = ++ (low), 11-30 cysts = +++ (medium), and > 30 = ++++ (high). Stool samples were kept frozen at -20ºC for genotyping study. Children who were positive for *Giardia* cysts were treated with metronidazole.

Genotypic Characterization of *G. duodenalis*

Giardia DNA was extracted using QIAmp DNA stool mini kit (QIAGEN, Germany) according to manufacturer instructions. Final

elutions of DNA were made in 100 µl of elution buffer. Genotypic characterization of *G. duodenalis* was determined using amplification of the small subunit ribosomal gene (SSU-rRNA) as previously described (Hopkins *et al*., 1997). PCR-RFLP was also performed using nested PCR of a 432 bp region of the glutamate dehydrogenase (*gdh*) gene. The primary primer pairs (forward primers; GDH1, GDH1a and reverse primer; GDH5s) with the condition for the first round PCR were used as previously described by Boontanom *et al*. (2010). The secondary PCR, using primers GDHeF/GDHiR, and the condition was described by Read *et al.* (2004). PCR-RFLP method of the *gdh* gene was conducted as previously described (Read *et al*., 2004). 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). PCR-positive products were sent to Macrogen Inc., Seoul, South Korea for DNA purification and sequencing. Nucleotide sequences were determined with Sequencer program (Gene Codes Corporation, Inc., Ann Arbor, MI). The genotype of *G. duodenalis* from each specimen was confirmed by the homology of the sequenced PCR product to the published sequences in GenBank, i.e., GenBank accession number; L40510 (*G. duodenalis* assemblage AII), AF069059 (*G. duodenalis* assemblage BIII) and L40508 (*G. duodenalis* assemblage BIV) by multiple alignments in ClustalX version 1.81 for Windows. The GenBank accession numbers assigned to the sequences determined in this study are as follows: assemblages AII, HM748028, HM748029, HM748030; BIII, HM747999; BIV, HM747981, HM747962, HM747963, HM747968, HM747969, and HM747970.

Data Analysis

The association between *G. duodenalis* infection and the characteristics of hilltribe children was assessed by Chi-square or Fisher's Exact test with a 95% confidence interval. All the analyses were conducted using Stata/SE for Windows, version 9.2 (StataCorp LP, College Station, TX, USA).

RESULTS

Characteristics of the enrolled children in the 2 districts are shown in Table 1. Of 765 school children, 589 (77.0%) were from Mae-Chaem District and 176 (23.0%) were from Hod District. The children were Karen (88.4%), Mong (6.7%), Lava (3.3%), Lesor (1.0%) and other (0.7%). The mean age was 8.2 ± 3.2 years (range, 1-16 years). The overall prevalence of giardiasis in these hilltribes was 5.2%. The prevalence of giardiasis in children from Mae-Chaem and Hod were 5.1% (30 in 589) and 5.7% (10 in 176), respectively. Giardiasis was observed in all age groups (1-12 years old). The prevalence of giardiasis was not significantly different among age groups. The highest prevalence was observed in children aged 4-6 years in Hod District; however, no significant difference was found ($p = 0.126$). In Mae-Chaem District, the prevalence of giardiasis in children aged over 12 years was significantly higher compared with the younger children ($p = 0.01$). *Giardia* infection was found only in the Karen of both groups from Hod and Mae-Chaem Districts while other tribes showed negative results.

Of 40 positive *G. duodenalis* samples collected from both groups, 28 (70%) were successfully amplified for genetic characterization. Table 2 shows the distribution of *Giardia* assemblages in these populations. Sequence analysis of SSU-rRNA gene revealed that 16 (57.1%) and 12 (42.9%) samples were assemblage A and assemblage B, respectively. Subgenotype of these isolates was also identified using PCR-RFLP of the *gdh* gene; of the 10 samples from Hod, 6 (60%), 3 (30%), 1 (10%), and 0 (0%) were identified as subgenotypes BIV, AII BIII, and AI, respectively. Of 18 samples from Mae-Chaem District, only 9 (50%) samples were successfully characterized by PCR-RFLP of the *gdh* gene of which 7 (77.8%) and 2 (22.2%) were subgenotypes AII and BIV, respectively.

Table 1. Characteristics of enrolled hilltribe children and prevalence of *Giardia duodenalis* infection

Characteristic	Mae-Chaem district			Hod district		
	Number	*G. duodenalis* Infection (%)	*P*	Number	*G. duodenalis* Infection (%)	*P*
Sex						
Male	302 (51.3)	14 (4.6)		83 (47.4)	6 (7.2)	
Female	287 (48.7)	16 (5.6)	0.709	92 (52.6)	4 (4.3)	0.521
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)	0.08	42 (24.0)	1 (2.4)	0.390
Tribe						
Karen	502 (85.2)	30 (6.0)		174 (98.9)	10 (5.8)	
Other	87 (14.8)	0 (0)	0.014*	2 (1.1)	0 (0)	1.000*
Total	589 (100)	30 (5.1)		176 (100)	10 (5.7)	

* Fisher's exact test

Table 2. Distribution of *Giardia* assemblages in the enrolled children

Assemblage	*SSU-rRNA*		*ghd*			
	A	B	AI	AII	BIII	BIV
Hod district	3 (30%)	7 (70%)	0 (0%)	3 (30%)	1 (10%)	6 (60%)
Mae-Chaem district	13 (72.2%)	5 (27.8%)	–	7 (77.8%)	–	2 (22.2%)
Total	16 (57.1%)	12 (42.9%)	0 (0%)	10 (52.6%)	1 (5.3%)	8 (42.1%)

The distribution of *Giardia* assemblage A and B infections was not different between sex and among age group (Table 3). However, children infected with assemblage A were significantly associated with loose to watery stools (p = 0.001). Additionally, children infected with *Giardia* assemblage B shed a significantly higher number of cysts than those infected with *Giardia* assemblage A (p = 0.019).

DISCUSSION

In this study of hilltribe children living in the remote areas of Chiang Mai, Northern Thailand, 5.2% of these children had giardiasis. The prevalence of giardiasis in the children of this region of Thailand was previously reported in a few studies, showing a similar range of prevalence as identified in the present study. In 1989, a study in primary school children in Chiang Mai showed a prevalence of 7.7% (Kasuya *et al.*, 1989), while in 2002, a survey in Nan, another province in Northern Thailand showed a prevalence of 5.5% (Waikagul *et al.*, 2002). Recent studies of giardiasis in school children of a rural community, Central Thailand, also showed a prevalence of 5.8–6.2% (Ratanapo *et al.*, 2008; Boontanom *et al.*, 2011). In contrast, a few studies in the urban, institutionalized Thai children showed a higher prevalence of giardiasis, which could be due to a higher rate of exposure from the crowded living and poor hygienic conditions (Ghosh *et al.*, 2000; Schuurman *et al.*, 2007). True prevalence of giardiasis could be

Table 3. Characteristics of 28 hilltribe children infected with *G. duodenalis* assemblages A and B

Characteristic	Assemblage found in infected children		
	A	B	*P*
Sex			
Male	9 (56.3)	5 (41.7)	
Female	7 (43.7)	7 (58.3)	0.704
Age (years)			
3–10	12 (75.0)	10 (83.3)	
> 10	4 (25.0)	2 (16.7)	0.673
Cyst shedding in stool			
1–2 cysts/ HPF	10 (66.7)	2 (16.7)	
3–10 cyst or more/ HPF	5 (33.3)	10 (83.3)	0.019*
Stool consistency			
Form	1(6.2)	8 (66.7)	
Loose/Watery	15(93.8)	4 (33.3)	0.001*

* Significant difference by Fisher's exact test

underestimated in the present and other studies that only used microscopy for the detection of *Giardia*. Higher prevalence could be obtained when more sensitive techniques such as ELISA or PCR, was used (Janoff *et al*., 1990; Saksirisampant *et al*., 2003). Among 5 tribes, the Karen comprised the majority of the enrolled subjects and was the only tribe that had giardiasis in both Mae-Chaem and Hod Districts. A recent study in the children living in Sangkhlaburi District, Kanchanaburi, Western Thailand, also showed a significantly higher prevalence of intestinal parasitic infections in the Karen (Wongstitwilairoong *et al*., 2007). Considering this similar finding in the different geographical areas, the high prevalence of intestinal parasitic infections in this tribe might be due to some specific risk behaviors. Unfortunately we have no information regarding the analysis of risk behaviors.

Since a small amount of unpreserved, long term stored fecal samples were used for genotypic characterization, only 70% and 50% of samples were successfully performed using PCR methods specific to the SSU-rRNA and the *gdh* genes, respectively. The distribution of *Giardia* assemblages varied in different geographical areas. Assemblage B was predominant in Brazilian, Egyptian, Argentinian and Thai children while assemblage A was the most prevalent in Peruvian children (Foronda *et al*., 2008; Kohli *et al*., 2008; Minvielle *et al*., 2008; Boontanom *et al*., 2011). The prevalence of assemblage A and B were equal in Albania and Cuba (Caccio *et al*., 2002; Pelayo *et al*., 2008). Using RAPD, two main genetic groups, RAPD 1 and RAPD 2, were observed from stool specimens collecting from different hospitals in Saudi Arabia (Shalaby *et al*., 2011). In this study, the prevalence of assemblage B was higher in the children of Hod District whereas assemblage A was more common in those of Mae-Chaem District. These two districts are approximately 240 km apart. From our observation, the school at Hod District is located in a dry mountainous area, having limited water access while that of Mae-Chaem District is in a forest area with adequate water supply. Differences of *G. duodenalis* assemblage among the studied populations could be due to different modes of transmission in each area, comprising person-to-person, waterborne, foodborne, or zoonotic transmission. Cattle are most commonly raised in Hod District, while dogs, pigs and cattle, were commonly found in Mae-Chaem District. Unfortunately, evidence of zoonotic transmission was not determined since fecal examination was not performed

in these animals. The distribution of giardiasis among age groups in these 2 populations was different, which might be explained by the different modes of transmission. In Mae-Chaem District, children aged over 12 years could be exposed to different sources of *G. duodenalis* since these children were more active. In this study, subgenotype BIII was noticed only in 1 child in Mae-Chaem District. Our results agree with previous studies in other countries showing that subgenotype BIII was uncommon in humans (Read *et al.*, 2004). In contrast, a high prevalence of subgenotype BIII has been reported in a previous study in Central Thailand (Tungtrongchitr *et al.*, 2010).

To date, the association between clinical symptoms and *Giardia* assemblages is still controversial. Unfortunately, we had no clinical information of these children. However, we have shown that assemblage A was significantly associated with loose or watery stool. Thus, further investigations in different groups of population are needed to make a definite conclusion of these contrary results. In the present study, children infected with assemblage B shed higher number of cysts than those infected with assemblage A, similar to the report by Kohli *et al.* (2008). It has been also demonstrated that a higher amount of genomic DNA of assemblage B was detected in stools (Haque *et al.*, 2005). A higher rate of cyst shedding of assemblage B could account for its high prevalence reported in populations of different geographical areas. A large number of cysts of assemblage B excreted in stools might help spread the transmitted cysts into the environment.

In conclusion, this study provides information on the prevalence of *G. duodenalis* infection in hilltribe children from Mae-Chaem (5.1%) and Hod (5.7%) Districts, northern Thailand. Sequence analysis revealed assemblage AII (52.6%), assemblage BIV (42.1%) and assemblage BIII (5.3%), respectively. Distribution of assemblage BIV was predominant in Hod District while assemblage AII was more common in children from Mae-Chaem District. A significantly higher number of cysts of assemblage B were shed in stools whereas assemblage A was significantly associated with loose/watery stool. Further studies on the epidemiology of giardiasis especially risk factors associated with genotyping would help to understand the nature of this disease, which will benefit the development of prevention and control strategies in this population.

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