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Incidence and risk factors of *Giardia duodenalis* infection in an orphanage, Thailand

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Abstract. A cohort study was performed to evaluate the incidence and risk factors of *Giardia duodenalis* infection in an orphanage in suburban area outside Bangkok, central Thailand. Stool specimens were examined for the presence of *G. duodenalis* in January 2007, May 2007 and January 2008. Of 892 stool specimens from 481 individuals, simple wet preparation, PBS ethyl-acetate sedimentation and PCR amplification of the SSU-rRNA gene were performed to detect *G. duodenalis*. Using PCR of the glutamate dehydrogenase gene and sequence analysis, *G. duodenalis* assemblages were identified. Associated risk factors were analysed using Fisher's exact test which revealed significant infection of *G. duodenalis* in boys and specific rooms where orphans aged 25-48 months old lived. Genotypic characterization of *G. duodenalis* revealed that assemblage A subtype AII was the most predominant found in orphans living in the specific rooms, thus the transmission was likely to occur via person-to-person. Other modes of transmission were less likely to occur. This study showed that the incidence rate of *Giardia* infection gradually decreased significantly after the implementation of health education and appropriate treatment of infected orphans.

INTRODUCTION

Giardiasis is an intestinal protozoal infection, caused by *Giardia duodenalis* commonly found in children (Thompson 2004; Feng *et al.*, 2013), especially those attending day care centers (Polis *et al.*, 1986), living in poor hygienic conditions (Saksirisampant *et al.*, 2003) or in community settings (Ratanapo *et al.*, 2008). Transmission occurs through faecal-oral contamination by person-to-person, waterborne, foodborne, and zoonotic transmissions. Infection may produce severe acute diarrhea in young children and impairs body weight gain, interfering with growth and development (Simsek *et al.*, 2004).

Molecular studies have revealed a complex species of *G. duodenalis* from which two major genetic assemblages, A and B, are recovered from humans (Mayrhofer *et al.*, 1995; Monis *et al.*, 1996, 1999, 2003). Other animal-specific groups include assemblages C, D, E, F, G (Monis *et al.*, 1996) and H (Lasek-Nesselquist *et al.*, 2010). Assemblage A has three distinct clusters; AI, AII and AIII (Cacciò *et al.*, 2008) while assemblage B consists of BIII and BIV (Monis *et al.*, 1996). To date, the predominant assemblage of *Giardia* varied considerably from country to country depending on study population.

In Thailand, the prevalence of giardiasis in children has been studied in different populations such as school children (Wongstitwilairoong *et al.*, 2007; Ratanapo *et al.*, 2008; Boontanom *et al.*, 2012; Saksirisampant *et al.*, 2012) and children in orphanages (Janoff *et al.*, 1990; Mungthin *et al.*, 2001; Saksirisampant *et al.*, 2003). The

prevalence varies from 5 to 37.7%. Among these, orphans had the highest prevalence of 37.7% (Saksirisampant *et al.*, 2003). Additionally, the highest prevalence was mostly among school children below 5 years old, and slightly higher in males than females (Ratanapo *et al.*, 2008). Our previous study of giardiasis in an orphanage, performed in Bangkok during 1999 to 2001 showed that the prevalence of *Giardia* was 12% in the first year of study and did not significantly decrease by the year 2001 (10.6%) (Mungthin *et al.*, 2003). Although all infected cases were treated with proper antiprotozoal drug, re-infection still subsequently occurred along these years. In 2006, this orphanage was moved out to a new place in suburban area where new buildings and houses with good facilities had been provided. Therefore, in the present study, we conducted a cohort study of intestinal parasitic infections emphasized on giardiasis in orphans to evaluate the incidence and risk factors of *G. duodenalis* infection using PCR and standardized questionnaires. In addition, genotypic characterization of *G. duodenalis*, isolated from these children was also investigated.

MATERIALS AND METHODS

Study design and study population

The study was conducted at an orphanage in suburban area outside Bangkok, Thailand. Stool examination was routinely performed to detect intestinal parasitic infections in orphans twice every year, in January and May. Orphans ages ranged from one month to 12 years old were enrolled into the study. A prospective cohort study of intestinal parasitic infections emphasized on *G. duodenalis* infection was conducted among these orphans during the routine screening for intestinal parasitic infections. Those who were negative for *G. duodenalis* in stool examination in January 2007 were enrolled into a follow-up survey to identify incidence and risk factors of *G. duodenalis* infection in May 2007. Those who were negative for *G. duodenalis* in May 2007 were subsequently enrolled into the second follow-up survey of *G. duodenalis* infection in January 2008. Inclusion criteria included orphans whose stools were collected at the time of study. Orphans whose stools could not be collected were excluded from the study. Additionally, a number of orphans who were adopted by foster parents during the time of study were considered as dropout cases. The child caretakers in each house were asked to complete the standardized questionnaires of their responsible orphans. The information included age, sex, weight, height, HIV status, the date of stool sample collected and the present illness (i.e. fever, nausea, diarrhea, abdominal pain) were recorded. The research protocol was approved by the Ethical Committee of the Royal Thai Army, Medical Department. All *Giardia*- infected individuals were treated with a single dose of Tinidazole 50 mg/kg.

Stool collection and examination

Stool specimens were examined for intestinal parasitic infections under light microscopy using simple wet preparation and PBS ethyl-acetate sedimentation techniques. All concentrated stools were washed 3 times with phosphate buffered saline and kept at -20 ºC for PCR assay.

DNA extraction of *G. duodenalis* in stool specimens

The concentrated specimens were extracted using FTA disk (Whatman, Bioscience, U.S.A.) (Nantavisai *et al.*, 2007). The washed FTA disks were used as DNA templates in PCR amplification. In addition, QIAmp stool mini kit (Qiagen, Germany) was used for DNA extraction for samples which gave negative PCR results using FTA disk. Final elutions of DNA were made in 100 µl of elution buffer instead of 200 µl as recommended by the manufacturer.

PCR amplification of the *SSU-rRNA* and the *gdh* genes

Extracted DNA was used for amplification of the small subunit of the ribosomal RNA gene (SSU-rRNA). The amplification of a 130 bp fragment of *G. duodenalis* SSU-rRNA gene was carried out by nested PCR using primers

RH11 (5'CAT CCG GTC GAT CCT GCC3'), RH4 (5'AGT CGA ACC CTG ATT CTC CGC CAG G3') and GiarF (5' GAC GCT CTC CCC AAG GAC3'), GiarR (5'CTG CGT CAC GCT GCT CG3') and the condition previously described (Hopkins *et al*., 1997).

Amplification of the *gdh* gene of *G. duodenalis* was carried out by nested PCR using specific primer pairs and sequence analysis for identification of the genotypes. To amplify the *gdh* gene, a primary External Forward Primer, GDH1 (5'ATC TTC GAG AGG ATG CTT GAG3'), GDH1a (5'ATC TTC GAG AAG GAT GCT TGA G3'), and External Reverse Primer, GDH5s (5'GGA TAC TTS TCC TTG AAC TC3') were used for primary PCR assay (Boontanom *et al*., 2010). For the secondary PCR, a 461 bp of the *gdh* gene was amplified using GDHeF (5'TCA ACG TYA AYC GYG GYT TCC GT3') and GDHiR (5'GTT RTC CTT GCA CAT CTC C3') with the condition previously described (Read *et al*., 2004).

Genotypic characterization

The PCR products were analyzed by 2% agarose gel electrophoresis, stained with ethidium bromide and then visualized on a UV transilluminator. Sequence analysis was conducted by 1st Base Pte. Ltd., Singapore. The genotype of *G. duodenalis* from each specimen was confirmed based on the homology of the sequenced PCR product to the published sequence in GenBank by multiple alignments in ClustalX version 1.81 (Thomson *et al*., 1997, Chenna *et al*., 2003). To identify assemblages and subgenotypes of *G. duodenalis*, sequence analysis of a 461 bp of the *gdh* gene was performed.

Statistical analysis

Fisher's exact test was used to compare proportion. Incidence rate per 100 person-years of observation were calculated for each category of demographic and the potential risk factor variables. The association between the potential risk factors and *G. duodenalis* infection were assessed using incidence rate ratios (IRR) and 95% confidence intervals (CI). To assess the independent association of the risk factors and *Giardia* infection, multivariate Poisson regression analysis was performed using Stata/SE version 9.2 (StataCorp LP, College Station, Texas, USA).

RESULTS

Characteristics of the enrolled subjects

A total of 481 participants from the three enrollments which comprised 364 orphans and 117 child caretakers were enrolled into the study. There were 205 males (42.6%) and 276 females (57.4%). Orphans' ages ranged from one month to 12 years. The median age was 21.2 months (7.7-99.4). Among these orphans, 68 out of 481 (14.1%) were HIV positive, of which 42 were male and 26 were female. All of these HIV-infected orphans lived together in House No. 4. There were 3 (1.0%) orphans who had experienced diarrhea. To examine the association of age group and the incidence of *G. duodenalis* infection, ages were categorized into 6 groups; 0-12 months, 12.1-24 months, 24.1-36 months, 36.1-48 months, 48.1-60 months and over 60 months.

Prevalence, incidence and incidence rate of *G. duodenalis*

Based on 892 stool specimens from 481 individuals, the overall prevalence of *G. duodenalis* was 6.0% (54/895). The highest prevalence (10.3%) was observed in January 2007, and significantly decreased in May 2007 (4.6%) and January 2008 (3.5%) (Table 1). There were 302 participants enrolled into a follow-up survey to identify the incidence and risk factors of *G. duodenalis* infection in May 2007, whereas 289 participants were subsequently enrolled into the second follow-up survey of *G. duodenalis* infection in January 2008. A pattern of the incidences was similar to that observed with the prevalences. As shown in Table 1, the incidence rate of *G. duodenalis* infection decreased from 1.04 (100 person-months) in May 2007 to 0.56 (100 person-months) in January 2008. Figure 1 shows both prevalence and incidence rate of *G. duodenalis* gradually decreased during the time of study.

Table 1. Prevalence, incidence and incidence rate of *G. duodenalis* from January 2007 to January 2008

	Prevalence N (%)	Incidence N (%)	Time (Person-months)	Incidence rate (100 person-months)	Total
January 2007	31 (10.3)	–	–	–	301
May 2007	14 (4.6)	9 (2.9)	862.5	1.04	302
January 2008	10 (3.5)	9 (3.1)	1604.8	0.56	289

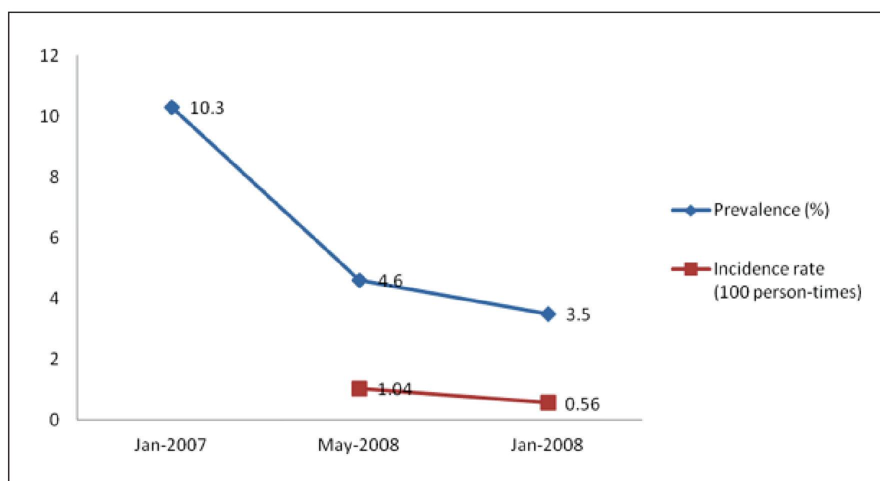

Figure 1. Prevalence and incidence rate (100 person-months) of *G. duodenalis* infection among orphans from January 2007 to January 2008

Comparison of the methods to detect *G. duodenalis* infection

The microscopic and SSU-rRNA PCR methods were used to detect *G. duodenalis* infection in 892 stool samples. Using microscopic method, 24 (2.7%) samples were positive for *G. duodenalis* cysts, whereas, 54 (6.0%) samples were positive in PCR amplification of SSU-rRNA. Therefore, PCR method was significantly more sensitive than conventional microscopy (χ^2 =116.8, p<0.001).

Genotypic characterization of *G. duodenalis* in orphans

PCR amplification of the *gdh* gene was successful in 36 (66.7%) out of 54 *Giardia* positive samples. Sequencing of the *gdh* gene showed that assemblage A subtype AII was the most predominant (26, 47.2%) followed by assemblage B subtype BIV (10, 27.8%). Sequences of the *gdh* gene were deposited in the GenBank database (AII: HM748031-HM748040; BIV: HM747987, HM747989-HM747991, HM747994-HM747997). Distribution of the subtypes is shown in Table 2.

Characteristics of the enrolled orphans and incidence of *G. duodenalis*

As shown in Table 3, the incidences of *G. duodenalis* infection were significantly

Table 2. Subgenotypes of *G. duodenalis* using PCR of the *gdh* gene and nucleotide sequence analysis

Assemblage (Subtype)	January 2007 N (%)	May 2007 N (%)	January 2008 N (%)
A(I)	–	–	–
A(II)	13 (68.4)	5 (55.6)	8 (100.0)
B(III)	–	–	–
B(IV)	6 (31.6)	4 (44.4)	–
Total	19 (100.0)	9 (100.0)	8 (100.0)

Table 3. Characteristics of the enrolled subjects and incidence of *G. duodenalis*

Characteristics	Enrolled subjects	No. of *G. duodenalis* infected person (%)	*P* value
Age (months)			
$\leq$ 12.0	83	0 (0.0)	
12.1-24.0	37	1 (2.7)	
24.1-36.0	31	8 (25.8)	
36.1-48.0	20	4 (20.0)	
48.1-60.0	7	2 (28.6)	
> 60.0	96	2 (2.1)	<0.001[a*]
House			
House 1	16	1 (6.3)	
House 2	19	0 (0.0)	
House 3	32	2 (6.3)	
House 4	57	1 (1.8)	
House 5	30	8 (26.7)	
House 6	23	4 (17.4)	
House 7	21	0 (0.0)	
House 8	24	0 (0.0)	
House 9	17	0 (0.0)	
House 10	22	1 (4.5)	
House 11	1	0 (0.0)	0.001[a*]
Sex			
Female	155	4 (2.6)	
Male	121	13 (10.7)	0.010[a*]
HIV-infection			
No	224	16 (7.1)	
Yes	52	1 (1.9)	0.211[a]
Diarrhea			
No	42	17 (40.5)	
Yes	7	0 (0.0)	0.080[a]

a: Fisher exact test * Significant difference at *P* value < 0.05

different among sex (Fisher's exact test =7.806, p = 0.010), age group (Fisher's exact test =33.602, p = <0.001), and house number (Fisher's exact test =22.986, p = 0.010). There was no statistically significant difference among those with HIV status and diarrhea.

Risk factors of *G. duodenalis* infection

Univariate and multivariate analysis of risk factors associated with *G. duodenalis* infection are shown in Table 4. Univariate analysis showed that age group, sex and house number were associated with *G. duodenalis* infection. After adjusted for sex, age group, house number, diarrhea and HIV infection, multivariate logistic regression analysis showed that orphans who lived in House No.5 and House No.6 had 6.2 (IRR= 6.2, 95%CI = 2.0-19.1) and 4.6 (IRR=4.6, 95%CI = 1.2-17.1) times greater risk of acquiring *G. duodenalis* than those who lived in other houses, respectively. In addition, male had 4.5 (IRR= 4.5, 95%CI = 1.4-13.9) times greater risk than female to acquire the infection. There was no significant association between *G. duodenalis* infection and HIV infection.

DISCUSSION

In the present study, a cohort study of *G. duodenalis* infection was conducted among orphans who lived in an orphanage located at Babies' Home in a suburban area of

Table 4. Univariate and Multivariate analysis for risk factors of *G. duodenalis* infection

Characteristics	No. positive	Person-months of follow up	IR (100 person-months)	IRR (95%CI)			
				Crude	***P*** value	adjusted	***P*** value
Age group (months)							
≤ 24.0	1	952.3	0.1	1			
24.1-60.0	15	567.6	2.6	25.2 (3.9-1059.4)	<0.001*		
> 60.0	2	935.3	0.2	2.0 (0.1-120.1)	0.615		
Sex							
Female	4	1409.6	0.3	1		1	
Male	14	1057.7	1.3	4.7 (1.5-19.6)	0.003*	4.5 (1.4-13.9)	0.009*
House							
Others	6	1836.5	0.3	1		1	
No5.	8	265.1	3.0	9.2 (2.8-32.9)	<0.001*	6.2 (2.0-19.1)	0.002*
No.6	4	234.3	1.7	5.2 (1.1-22.0)	0.023*	4.6 (1.2-17.1)	0.023*
HIV infection							
No	17	1954.7	0.9	1		1	
Yes	1	512.6	0.2	0.2 (0.01-1.4)	0.102	0.3 (0.04-2.9)	0.318

Data were adjusted for age group, sex, room group and HIV infection.
IR = Incidence Rate; IRR = Incidence Rate Ratio; 95%CI = 95% Confidence Interval;
*Significant difference at *P* value < 0.05

Bangkok. This orphanage composed of separated houses and clean rooms with good facilities. Using microscopic method to examine *Giardia* infection in stool specimens, the prevalence could be underestimated due to its low sensitivity, compared with other sensitive techniques such as ELISA and PCR methods (Schuurman *et al.*, 2007; Al-Saeed *et al.*, 2010). Thus, PCR technique was performed on all samples to demonstrate the true prevalence and incidences of *Giardia* infection in January 2007, May 2007 and January 2008. Our study showed that microscopic method could reveal only 24 (2.7%) infected subjects, whereas by using PCR method, positive subjects significantly increased to 54 (6.1%). Thus, identifying true risk of *G. duodenalis* infection could benefit prevention and control measures in this setting. To eliminate the source of transmission, proper treatment as well as providing health education to child caretakers could reduce the infection. The study showed that treatment of infected orphans especially those who had very low numbers of cyst shedding could eliminate the source of transmission. Both prevalence and incidences of *G. duodenalis* infection were gradually decreased during the period of study. A pattern of the incidence was also similar to that observed with the prevalence. The incidence rate or newly infected *Giardia* cases were 1.04 per 100 person-months in May 2007 and subsequently decreased to 0.56 per 100 person-months in January 2008.

The prevalence of *G. duodenalis* infection in these orphans were lower than those from many previous studies (Janoff *et al.*, 1990; Mungthin *et al.*, 2001; Saksirisampant *et al.*, 2003). In addition, no significant association was found between *G. duodenalis* infection and nutritional status (data not shown). Most infected orphans were asymptomatic, similar to those of previous reports (Thompson *et al.*, 1993; Mungthin *et al.*, 2001).

The highest *Giardia* prevalence among children aged 25-48 months old, found at Houses No. 5 and No. 6, was observed, which agreed with previous studies performed in Thailand (Mungthin *et al.*, 2001; Saksirisampant *et al.*, 2003), Guatemala, Canada, Portugal, the United States of America, and Brazil (Farthing *et al.*, 1986; Isaac-Renton *et al.*, 1992; Orlandi *et al.*, 2001; Santos *et al.*, 2012). This might be explained by the fact that children aged 25-48 months old have more active movement and more independent eating habits when compared to those in the first year of age.

However, they still have poor toilet training and poor food-handling hygiene that could favor the spread of *Giardia* cysts. This study also showed that boys had greater risk than girls to get the infection.

To date, the predominance of *Giardia* assemblage varies considerably from country to country depending 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) while in Argentina, assemblage B was mostly found in both adults and children (Minvielle *et al.*, 2008). In Thailand, the study of molecular characterization of *G. duodenalis* in a closed community was reported for which the distribution of *Giardia* assemblages also varied among study populations. The predominant genotype was assemblage A, subtype AII followed by assemblage B subtype BIV and were identified in primary schoolchildren in a rural community, Chachoengsao Province, central Thailand (Boontanom *et al.*, 2011). However, hilltribe children at two different areas in Northern Thailand harboured different assemblages. Assemblage B subtype BIV was found predominantly in children from Hod District while assemblage A subtype AII was more common in children from Mae-Chaem District (Saksirisampant *et al.*, 2012). The findings of our study also presented a higher prevalence of assemblage A than B. The infections of both assemblage A and assemblage B were detected in House No. 5 whereas those in House No. 6 revealed only a human-specific subtype, assemblage A subtype AII. The higher prevalence of subtype AII revealed an anthroponotic origin of the infection, thus it could be postulated that the transmission was person-to-person. The transmission could have occurred via faecal-oral route among high risk groups of orphans. Other modes of transmission such as waterborne, foodborne, and animal-to-person transmission were less likely to occur. Assemblage B subtype BIV, a zoonotic potential subtype, was the second most common subtype found in orphans. Zoonotic transmission of subtype BIV was less likely to occur since pets were not allowed in this setting. The infection might have come from either newly admitted orphans or child caretakers. Additionally, we found three child caretakers were infected with *Giardia*. Only one of the child caretaker had infection of assemblage B subtype BIV. Three infected children who were re-infected during the study also revealed the same subtype AII in both their first and second infections. We also found infections of both assemblage A and B in HIV positive children. Moreover, infections of both subtype AII and subtype BIV in orphans were found in January 2007 and May 2007, however, infection of only subtype AII was found in January 2008. The transmission of *Giardia* assemblage was not associated with seasonal variation.

This study provided information of significant risk factors associated with *Giardia* infection in an orphanage, namely, age group, sex and house number which could lead to more effective prevention and control programmes of giardiasis in this population.

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