Incidence and Risk factors of *Blastocystis* infection in Orphans at the Babies' Home, Nonthaburi Province, Thailand

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Background: *Blastocystis infection is one of the most common intestinal protozoan infections reported in Thai population of all age groups for which epidemiological information is important to understand patterns of transmission for developing methods of prevention and control for each specific group. The authors aimed to study prevalence, incidence and risk factors associated with Blastocystis infection in orphans and childcare workers. Additionally, subtypes of Blastocystis were identified.*
Material and Method: *A retrospective cohort study of Blastocystis sp. was conducted in orphans aged less than 5 years and their childcare workers at Babies' Home, Nonthaburi Province, Thailand. A base line survey was conducted in December 2009 and a follow-up survey was conducted in April 2010. A total of 336 and 331 stool samples were collected. Blastocystis infection was examined using short-term in vitro cultivation in Jones's medium supplemented with 10% fetal calf serum. To analyze subtypes of Blastocystis sp., PCR-RFLP of the small subunit ribosomal RNA gene was performed.*
Results: *The prevalence of Blastocystis infection in December 2009 and April 2010 were 8.1% and 13.3%, respectively. The incidence rate of Blastocystis infection was 1.6/100 person-months. Subtype analysis of Blastocystis sp. in December 2009 and in April 2010 showed that subtype 3 was the most predominant (76% and 76%), followed by subtype 1 (16% and 20%), and unidentified subtype (8% and 4%), respectively. Subtype 3 is of human origin, thus person-to-person transmission is considered a major route of Blastocystis infection in this population.*
Conclusion: *Person-to-person transmission of Blastocystis infection in orphans living in the same house had been proposed, thus the prevalence and incidence of Blastocystis infection could be used to reflect the hygienic condition in the orphanage. Infection prevention and control practice can be effectively implemented.*

Keywords: *Blastocystis infection, Blastocystis sp., Subtype, Orphans*

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Blastocystis infection is an intestinal protozoan infection with a worldwide distribution especially in developing countries. The prevalence of *Blastocystis* infection varies among countries and communities. The risk association of *Blastocystis* infection is animal exposure and consumption of contaminated food or water; thus, a high prevalence of *Blastocystis* infection has been reported more in developing countries than in developed countries. A low prevalence of *Blastocystis* infection was reported in Japan (0.5 to 1%)[1,2], Singapore (3.3%)[3] while a high prevalence was found in Indonesia (60%)[4], 1.9 to 32.6% in China[5] and 1.04 to 18.3% in Thailand[6-9]. To date, using nucleotide sequence analysis, 17 subtypes of *Blastocystis* sp. can be classified[10-12] of which nine subtypes (subtypes 1 to 9) of *Blastocystis* have been reported in humans. Of these subtypes, subtype 3 has been classified as a human subtype. Subtype analysis of *Blastocystis* also revealed different prevalence of subtypes in each country. Subtype 3 is the most predominant reported in many countries including Bangladesh, China, Denmark, Egypt, Germany, Greece, Japan, Pakistan, Singapore, and Turkey[13], whilst subtype 1 is predominant in Thailand and different when compared to those of other countries[6,14].

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Since *Blastocystis* sp. is commonly identified in Thai populations of all age groups, epidemiological data are important for understanding patterns of transmission for developing methods of intervention for each specific group. Our previous report on incidence and risk factors of *Blastocystis* infection was conducted in an orphanage located in Bangkok, Thailand from April 2003 to April 2004[15]. Until 2006, all orphans were transferred to a new orphanage, located at a suburban area in Nonthaburi Province. Thus, our objectives aimed to study the prevalence, incidence, and risk factors of *Blastocystis* sp. in orphans and their childcare workers at the new Babies' Home, Nonthaburi Province, Thailand in December 2009 and April 2010. In addition, subtype identification of *Blastocystis* sp. was also performed in the present study.

Material and Method

Study population

A retrospective cohort study of *Blastocystis* infection was conducted at Babies' Home, Nonthaburi Province, Thailand. This orphanage was opened in 2006 after the Babies' Home located in Bangkok was closed. All orphans and their childcare workers were transferred to the new place. Each of 10 houses could accommodate 20-40 orphans of different ages who were less than 5 years old. Of the houses, one specific house was designated for those HIV-positive. House number 11 was assigned to orphans, aged 5-12 years old, who had been living in the orphanage. Orphans of each house were taken care of by their own 1-2 childcare workers. One specific house was used to prepare food and milk. The childcare workers in each house were also enrolled in the present study and were asked to collected stool samples as well as completed the questionnaires. The research protocol was reviewed and approved by the Ethics Committee of the Royal Thai Army Medical Department.

Stool collection and examination

Blastocystis infection was detected using short-term in vitro cultivation in Jones's medium supplemented with 10% fetal calf serum[16,17]. The cultures were incubated at 37°C for 48-72 hours and then examined under a light microscope.

Polymerase chain reaction and restriction fragment length polymorphism (PCR- RFLP)

Genomic DNA of *Blastocystis* sp. was extracted using Favorprep™ stool DNA isolation mini kit following the manufacturer's protocol. The extracted DNA was frozen at -20°C until use. PCR was performed to amplify the SSU rDNA gene of *Blastocystis* sp. using the forward primer SR1F (5'-GCTTATCTG GTTGATCCTGCCAG TAGT-3') and the reverse primer SR1R (5'-TGATCCTTCCGCAGGTTCACCTA-3') as described by Yoshikawa et al[18]. These primer pairs amplified approximately 1780 bp PCR product of the SSU-rRNA gene. The PCR condition was performed with 30 cycles of 94°C for 60 sec, 56°C for 60 sec, and 72°C for 90 min, after an initial denaturation at 94°C for 5 min. The DNA was treated through electrophoresis in 2% agarose gels and 1xTris-borate-EDTA buffer at 100 volts for 50 min. The PCR products were digested using two restriction enzymes; *Hinf*I and *Rsa*I (Gibco, BRL, Gaithersburg, Md.), and separated by 2% agarose gel electrophoresis and then visualized under UV light. They were then documented on high-density printing paper using a UV save gel documentation system I (Uvitech, UK).

Sequencing of the SSU-rRNA gene

To confirm the subtypes, partial sequences of SSU-rRNA of *Blastocystis* sp. were conducted by 1st Base Pte. Ltd., Singapore. Chromatograms were manually checked and edited using Sequencher demo version 4.0.5. Multiple alignment was performed to align the validated partial sequences of the SSU-rRNA gene from samples with *Blastocystis* sp. in GenBank database, using program Clustral X version 1.81[19,20].

Questionnaire

To determine the risk factors and outcomes of *Blastocystis* infection, standardized questionnaires concerning demographic data, HIV status, and house number were included. Their nutritional status was determined using criteria from the Institute of Nutrition, Mahidol University (INMU) Thai Growth Study[21].

Statistical analysis

To determine the incidence of and risk factors for *Blastocystis* infection, incidence was defined as the number of new *Blastocystis* infections occurring during the observation period. The midpoint-estimated date of infection for incident cases was taken between the last negative result and first positive result. Risk factors were analyzed using incidence rate ratios and 95% confidence intervals. Chi-square test or Fisher's exact test was used to compare proportions. Poisson regression using STATA/SE for Windows version 9.2 (StataCorp LP, College Station, TX) was performed for univariate and multivariate analysis to assess the

independent association of the risk factors and *Blastocystis* infection. A *p*-value <0.5 was considered statistically different.

Results

Study population

A baseline survey of *Blastocystis* infection was carried out in an orphanage in December 2009 and was followed-up in April 2010. A total of 412 participants were enrolled in the baseline study comprising of 297 orphans and 115 childcare workers. Of these, 178 were male and 234 were female. Forty-one orphans were HIV-positive. The median ages of the enrolled orphans and childcare workers were 1.4 years (0.6 to 3.1years) and 43.2 years (31.9 to 51.0 years), respectively.

Those individuals who were negative for *Blastocystis* sp. from the baseline survey were enrolled in a follow-up survey in April 2010. In all, 227 individuals or 55.1% of the baseline population participated in the follow-up survey. Of these, 168 were orphans and 59 were childcare workers. Those were 103 males and 124 females. Of 168 orphans, 30 were HIV-positive. The median ages of the enrolled orphans and childcare workers were 1.9 years (1.2 to 3.6 years) and 41.9 years (30.7 to 50.9 years), respectively. All HIV-positive orphans were prescribed antiretroviral therapy (i.e. zidovudine and didanosine).

Characteristics of Blastocystis infection in orphans

The characteristics of the participants are shown in Table 1. *Blastocystis* infection was significantly different among age groups ($p<0.001$), and houses ($p = 0.002$). The present study classified the ages into three groups (<3 years, 3-12 years, >12 years) and showed that those who were 3 to 12 years and greater than 12 years showed a significant difference of *Blastocystis* infection compared with those who were less than 3 years old. No significant difference was found among gender, HIV status, nutritional status or infected childcare workers considerations in the house.

Prevalence and incidence of Blastocystis infection

The overall prevalence of *Blastocystis* infection were 11.1% in December 2009 and 13.0% in April 2010, respectively. The incidence of *Blastocystis* infection include 16 cases (7.0%) with an incidence rate of 1.6/100 person-months.

Subtype analysis using PCR-RFLP

Restriction digestion of PCR products of 1,780 bp using two restriction enzymes, *Hinf*I and *Rsa*I, revealed subtypes 1, 3 and an unidentified subtype. In December 2009, of 35 positive *Blastocystis* samples, 25 (71.4%) could be amplified using PCR of which 4 (16%) were identified as subtype 1, 20 (80%) were identified as subtype 3 and 2 (8%) were identified as an unidentified subtype. In April 2010, of 39 positive *Blastocystis* samples, 25 (64.1%) could be amplified using PCR of which 5 (20%) were identified as subtype 1, 20 (80%) were identified as subtype 3 and 1 (4%) was identified as an unidentified subtype.

Risk factors of Blastocystis infection

Univariate analysis showed that no significant difference of *Blastocystis* infection between sex, HIV status, nutritional status and children who lived in houses where childcare workers were infected with *Blastocystis* sp., as shown in Table 2. However, after adjusting for gender, HIV status, nutritional status and other variables in the model, multivariate Poisson regression analysis showed that increasing risk of acquiring *Blastocystis* infection occurred when age increased. The risk of acquiring *Blastocystis* sp. increased at a rate of 1.04 for each additional year of the orphan's age (IRR = 1.04, 95% CI, 1.00-1.08). Additionally, House No. 5 (IRR = 10.6; 95% CI = 2.3-53.2) and House No. 11 (IRR = 14.9; 95% CI = 3.8-69.4) were independently associated with increased risk of acquiring *Blastocystis* infection than other houses.

Discussion

A previous study of incidence and independent risk factors of *Blastocystis* infection had been performed in orphans in Bangkok, which could bring up some strategies for prevention and control of *Blastocystis* infection in the orphanage[15]. In the present study, the prevalence of *Blastocystis* sp. in orphans living in a new orphanage, Nonthaburi Province was performed in December 2009 and the incidence was conducted five months apart in April 2010. In this study, the follow-up time was longer when compared with our previous study conducted every two consecutive months on the incidence of *Blastocystis* infection. The present study also showed that *Blastocystis* sp. was still one of the most common enteric protozoan parasites found in orphans. The prevalence of *Blastocystis* sp. in December 2009 (11.1%) and of April 2010 (13%) were still high requiring continuous prevention and control measures. Our results showed no significant difference of *Blastocystis* infection among gender, HIV status and nutritional status considerations. The HIV-positive orphans were

Table 1. Characteristics and incidence of *Blastocystis* infection, Babies' Home, in December 2009 and in April 2010

Characteristic	No. enrolled subjects	No. (%) infected subjects	*p*-value
Sex			0.797
Male	103	8 (7.8)	
Female	124	8 (6.5)	
Age group, years			<0.001*
<3	113	0 (0.0)	
3-12	55	11 (20.0)	
>12	59	5 (11.1)	
Study population			0.768
Orphans	168	11(6.5)	
Childcare workers	59	5 (8.5)	
House No.			0.002[a]
1 (36-60 months)	3	0 (0.0)	
2 (new born to 8 months)	10	0 (0.0)	
3 (new enrolled)	11	0 (0.0)	
4 (HIV-infected children)	27	1 (3.7)	
5 (36-48 months)	24	5 (20.8)	
6 (24-32 months)	26	1 (3.8)	
7 (new born to 8 months)	24	0 (0.0)	
8 (8-12 months)	21	0 (0.0)	
9 (12-18 months)	25	1 (4.0)	
10 (18-24 months)	26	0 (0.0)	
11 (5-12 years)	25	7 (28.0)	
Food & milk preparation house	3	0 (0.0)	
HIV infection			1.000[a]
No	25	1 (4.0)	
Yes	30	1 (3.3)	
Nutrition status			1.000[a]
Normal	147	11 (7.5)	
Under nutrition	35	2 (5.7)	
Over nutrition	45	3 (6.7)	
Infected childcare workers in the house			0.056
No	123	5 (4.1)	
Yes	104	11 (10.6)	

[a] Fisher exact test

provided with special health care including good hygiene, thus a low incidence rate ratio was observed (IRR = 0.7). In the present study, age groups between 3-10 years old and greater than 10 years were significantly different regarding acquiring *Blastocystis* sp. than those who were less than 3 years old. The result of this study was consistent with the previous one performed in the Bangkok orphanage[15] that the risk for acquiring *Blastocystis* sp. was found in orphans aged more than 3 years and increased at a rate of 1.04 for each additional year. House No. 11, an extra house provided for a number of orphans, aged 5 to 12 years, who still lived in the orphanage, showed the highest incidence rate of infection and was independently associated with increased risk of acquiring *Blastocystis* infection than other houses. This was similar to those who lived in House No. 5, provided for orphans aged 3-4 years, who also showed higher risk of getting the infection than those who were less than 3 years. Person-to-person transmission of *Blastocystis* infection could easily occur in the institutions where a large number of persons have been living and share facilities together. Orphans, aged 3-10 years and older, might have acquired the infection while they were playing together or sharing personal items with infected individuals living in the same house such as toys, clothing, bedding and others. These contaminated items might be put into the mouth. Since infected individuals shed the infected cysts in their stool, person-to-person transmission through the fecal-oral route usually occurred among

Table 2. Univariate and multivariate analysis of risk factors for*Blastocystis* infection

Characteristics	No. positive for *Blastocystis*	Person-months of follow-up	IR (100 person-months)	IRR (95% CI)			
				Crude IRR (95% CI)	*p*-value	Adjusted IRR (95% CI)	*p*-value
Sex							
Male	8	454.9	1.8	1		1	
Female	8	568.5	1.4	0.8 (0.3- 2.4)	0.661	0.4 (0.1-1.6)	0.183
Age (years)	16	1,023.5	1.6	1.02 (0.99-1.04)	0.155	1.04 (1.00-1.08)	0.035*
Nutritional status							
Normal nutrition	11	653.4	1.7	1		1	
Abnormal nutrition	5	370.0	1.4	0.8 (0.2-2.5)	0.708	0.6 (0.2-1.8)	0.337
HIV infection							
No	1	115.9	0.9	1			
Yes	1	135.0	0.7	0.9 (0.01- 67.4)	0.924		
House No.							
Other	4	828.2	0.5	1			
5	5	98.0	5.1	10.6 (2.3- 53.2)	0.001*		
11	7	97.2	7.2	14.9 (3.8-69.4)	<0.001*		
Blastocystis infection in child care workers in the house							
No	5	569.9	0.9	1		1	
Yes	11	453.6	2.4	2.8 (0.9-10.1)	0.056	2.7 (0.9-7.7)	0.069

Data were adjusted for sex, HIV status, nutrition status and other variables in the model. IR= incidence rate; IRR = incidence rate ratio; CI = confidence interval

members living in the same house. The childcare workers in the house who had *Blastocystis* infection could be an important asymptomatic carrier to orphans. The previous study showed that orphans living in the house where childcare workers had *Blastocystis* infection had higher risk to acquire infection[15]. After adjusting for confounders, the present study could not reveal the independent risk of *Blastocystis* infection in orphans having infected childcare workers in their house (IRR = 2.7; 95% CI = 0.9-7.7). This might be due to the follow-up time, which was longer than that of the previous study (5 months vs. 2 months). The previous study showed that time to clear a *Blastocystis* infection in orphans was 2.59 months[16]. However, the present study was not aimed at determining the time to clearance in these children.

Research studies reported that *Blastocystis* could be either asymptomatic or symptomatic in infected individuals. Pathogenicity of *Blastocystis* infection may be linked to specific subtype. Of 17 subtypes, subtype 3 was the most predominant reported in asymptomatic individuals and subtype 1 was found in symptomatic patients[22]. In addition, subtype 4 was linked to acute diarrhea[23]. More studies in different populations are needed to confirm the pathogenicity of these subtypes. However, subtyping alone does not predict pathogenicity since other factors might be involved, i.e., intra-subtype variation, host immunity, or other intrinsic factors in the host. In the present study, *Blastocystis*-infected orphans and childcare workers showed no gastrointestinal symptoms (data not shown). However, watery stool was observed in only one orphan for which *Blastocystis* infection might be the cause. Further study needs to be performed to elucidate symptoms caused by *Blastocystis* infection in young children.

In the present study, subtype identification was performed using PCR-RFLP to confirm the mode of transmission of *Blastocystis* sp. in this orphanage. Of 74 positive *Blastocystis* samples, only 50 (67.5%) samples successfully performed PCR-RFLP for subtype identification. Reduced PCR sensitivity could be due to a long preservation of the samples or fragmentation of DNA could have occurred during steps of DNA extraction. Subtype 3, a human subtype, was the most predominant subtype (76% and 76%), followed by subtype 1 (16% and 20%), found among these orphans and their childcare workers in December 2009 and in April 2010, respectively. This study showed a difference of subtypes predominant in orphans when compared with previous studies in Thailand. The present study showed that subtype 1 was predominantly found in school children and adults[14,15]. However, the result was similar to other reports performed in other countries, where subtype 3 was the most common[14]. In this orphanage, person-to-person transmission of subtype 3 and subtype 1 among orphans living in the same house was the most likely to occur. Other routes of transmission, *i.e.*, water borne and food borne, were less likely to occur since food and milk were prepared only in a food preparation house. In addition, the possibility of orphans acquiring *Blastocystis* of animal subtypes was less likely to occur since pets were not allowed in the orphanage. However, childcare workers were easily exposed to domestic animals and were more likely to get the infection than orphans were since they did not live in the orphanage.

In conclusion, the present study showed that person-to-person transmission of *Blastocystis* infection in orphans living in the same house was the most likely route, thus the prevalence and incidence of *Blastocystis* infection could be used to reflect the hygienic conditions in the orphanage. Good personal hygiene, including proper and frequent hand-washing is the best way to prevent person-to-person transmission of *Blastocystis* infection. For these young orphans, regular cleaning of clothing, bedding, toys and household items is recommended.

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Potential conflicts of interest

None.

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อุบัติการณ์ปัจจัยเสี่ยงและการติดเชื้อ *Blastocystis* ในบ้านเด็กอ่อน จังหวัดนนทบุรี ประเทศไทย

จิตรลดา บุญดิษฐ์, ดวงเนตร พิพัฒน์สถิตพงศ์, มฑิรุทธ มุ่งถิ่น, ปานจิต ธรรมศรี, พีรพรรณ ตันอารีย์, ทวี นาคหล่อ, เสาวนีย์ ลีละยูวะ

ภูมิหลัง: *Blastocystis sp. เป็นโปรโตซัวในลำไส้ที่พบบ่อยในคนไทยในทุกกลุ่มอายุ การศึกษาข้อมูลทางระบาดวิทยาของเชื้อมีความสำคัญ การศึกษาความชุกอุบัติการณ์และปัจจัยเสี่ยงของการติดเชื้อ Blastocystis sp. ในเด็กอ่อนและพี่เลี้ยงเด็กและแยก subtypes ของเชื้อ Blastocystis sp. ทำให้เข้าใจการแพร่กระจายของเชื้อเพื่อหาวิธีในการควบคุมและป้องกัน การติดเชื้อในกลุ่มอายุต่าง ๆ*

วัสดุและวิธีการ: *ได้ทำการศึกษาชนิด cohort ของการติดเชื้อ Blastocystis sp. ในเด็กอ่อนอายุน้อยกว่า 5 ปี ที่บ้านเด็กอ่อนปากเกร็ด จังหวัดนนทบุรี โดยเริ่มศึกษาข้อมูลพื้นฐานใน เดือนธันวาคม พ.ศ. 2552 และติดตามในเดือนเมษายน พ.ศ. 2553 โดยดำเนินการเก็บอุจจาระจำนวน 336 รายในครั้งแรกและเก็บอีกครั้งจำนวน 331 รายในการติดตามผล*

วิธีการตรวจวินิจฉัยทำโดยการเพาะเชื้อในหลอดทดลองชนิด Jone's medium ที่มีส่วนผสมของ fetal calf serum 10% และใช้วิธี PCR-RFLP กับ small subunit ribosomal RNA gene สำหรับการแยก subtypes ของเชื้อ

ผลการศึกษา: *ความชุกของเชื้อ Blastocystis sp. ในเดือนธันวาคมและเดือนเมษายน เท่ากับ 8.1% และ 13.3% ตามลำดับ อุบัติการณ์เท่ากับ 1.6/100 person-months การแยก subtypes ของเชื้อในเดือนธันวาคม พ.ศ. 2552 และติดตามในเดือนเมษายน พ.ศ. 2553 พบดังนี้ subtype 3 พบมากที่สุดโดยตรวจพบ 76% ทั้งสองครั้งตามด้วย subtype 1 (16% และ 20%) และ unidentified subtype (8% และ 4%) ตามลำดับ เนื่องจาก subtype 3 เป็นชนิดที่พบในคนโดยเฉพาะดังนั้นการติดเชื้อ Blastocystis sp. ในสถานเลี้ยงเด็กแห่งนี้เป็นไปได้ที่จะเกิดจากการติดต่อระหว่างบุคคลมากที่สุด*

สรุป: *การติดต่อของเชื้อ Blastocystis ในเด็กเกิดระหว่างบุคคลมากที่สุด ดังนั้นการศึกษาความชุกและอุบัติการณ์สามารถแสดงสุขอนามัยภายในสถานเลี้ยงเด็กได้ ซึ่งสามารถป้องกันและควบคุมการแพร่ของเชื้อได้*