

Identification of *Blastocystis* Subtype 1 Variants in the Home for Girls, Bangkok, Thailand

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Abstract. A cross-sectional study of *Blastocystis* infection was conducted to evaluate the prevalence, risk factors, and subtypes of *Blastocystis* at the Home for Girls, Bangkok, Thailand in November 2008. Of 370 stool samples, 118 (31.9%) were infected with *Blastocystis*. Genotypic characterization of *Blastocystis* was performed by polymerase chain reaction and sequence analysis of the partial small subunit ribosomal RNA (SSU rRNA) gene. Subtype 1 was the most predominant (94.8%), followed by subtype 6 (3.5%) and subtype 2 (1.7%). Sequence analyses revealed nucleotide polymorphisms for *Blastocystis* subtype 1, which were described as subtype 1/variant 1, subtype 1/variant 2. *Blastocystis* subtype 1/variant 1 was the most predominant infection occurring in almost every house. The results showed that subtype analysis of *Blastocystis* was useful for molecular epidemiological study.

INTRODUCTION

Blastocystis is a common intestinal parasite found in humans and a wide range of animals.^{1–6} Individuals infected with *Blastocystis* were mostly asymptomatic, whereas some patients presented with acute or chronic gastrointestinal illness.^{7–9} *Blastocystis* infection has been reported from both developed and developing countries.¹⁰ In Thailand, epidemiological studies of *Blastocystis* infection showed the prevalence of 10–40% in different populations.^{3,11–15} Using molecular methods, extensive genetic diversity was detected among *Blastocystis* populations.^{16–18} Recently, a consensus terminology for subtypes of *Blastocystis* was developed on the basis of small subunit ribosomal RNA (SSU rRNA) gene analysis for which at least 13 subtypes have been characterized from humans and animals.^{5,6,19} These subtypes are not host-specific and cross-infectivity may occur among humans and animals. Subtype characterization of *Blastocystis* is important for epidemiological studies that could help identify sources and potential routes of transmission of *Blastocystis* in each particular area. Subtype 3 is the most common subtype found in humans, however in Thailand subtype 1 appears to predominate followed by subtypes 2, 3, 5, 6, and 7.^{3,10,14,20}

Our recent study showed that *Blastocystis* was common in an orphanage for young children in Bangkok and possibly transmitted from person to person.¹⁵ The aim of this study was to determine the prevalence and risk factors of *Blastocystis* infection in the Home for Girls, an orphanage for girls with different age groups and living environments and to characterize subtypes of *Blastocystis* isolated from these girls to understand the epidemiology of *Blastocystis* infection in this population.

MATERIALS AND METHODS

Study population. A cross-sectional study of *Blastocystis* infection was conducted at the Home for Girls, Bangkok, in November 2008. The Home for Girls was established to pro-

vide welfare for abused and neglected girls. The study population consisted of 463 female participants between 5 and 25 years of age. The accommodation was arranged in cottagetype housing and had a capacity for 13 to 27 girls with different age groups in one house. There were 19 houses located next to each other having its own yard, rooms, kitchen, and cooking facilities. Drinking water was provided in each house using a filtered device. The institute did not allow these girls to have pets. However, dogs and cats were often seen around the area. The research protocol was reviewed and approved by the Ethical Committee of the Royal Thai Army Medical Department. Informed consents were obtained from the participants and/or the guardian before enrollment.

Collection and processing of fecal samples. A stool specimen of each enrolled subject was collected and examined for *Blastocystis* by *in vitro* cultivation using Jones' medium supplemented with 10% horse serum.²¹ Samples were incubated at 37°C for 2–3 days and examined for *Blastocystis* by light microscopy. Stool specimens of six domestic dogs were also processed by the same method. All positive cultured samples were washed twice with phosphate buffered saline and extracted for DNA using QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. The extracted DNA of each sample was kept frozen at –20°C until used.

Collection and processing of drinking water samples. Six liters of drinking water samples were collected from every house for the detection of *Blastocystis* using *in vitro* cultivation and polymerase chain reaction (PCR) methods. These water samples were filtered using 0.22- μ m filters. Each filter paper was then cut into two pieces for cultivation in Jones' medium and for genomic DNA extraction. The DNA extraction was performed using UltraClean Water DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA), according to the manufacturer's instructions. The extracted DNA of water samples was kept frozen at –20°C until used.

PCR of SSU rRNA region. Extracted DNA of *Blastocystis* from stool specimens and drinking water was amplified using the nested PCR technique. Primary and secondary PCR were carried out using primers and conditions described by Clark and others¹⁷ and Böhm-Gloning and others,¹⁶ respectively. The 1,100 bp of secondary PCR product was visualized by gel electrophoresis using 2% agarose gel and documented on

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high density printing paper using Uvisave gel documentation system I (Uvitech, Cambridge, UK).

DNA sequencing and subtype analysis. To subtype and characterize genetic diversity of each subtype, all PCR products from 58 samples were directly sequenced and chromatograms were validated using BioEdit version 7.0.1 (Isis Pharmaceuticals Inc., Carlsbad, CA). Subsequently, 1,005 bp of nucleotide sequences of the SSU rRNA gene of *Blastocystis* obtained in this study were multiple aligned with a set of 44 other *Blastocystis* isolates retrieved from the GenBank database including representatives of all known *Blastocystis* subtypes using ClustalX version 2.0.12.²² Phylogenetic analysis was carried out using MrBAYES version 3.1.2.²³ Bayesian analyses of the SSU rRNA data set were performed using the GTR (general time reversible) + Γ (gamma distribution of rates with four rate categories) + (proportion of invariant sites) model of sequence evolution. 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 3.1.2²³; all sequences obtained from this study were submitted to GenBank under the accession no. JQ665844–JQ665872.

Questionnaire. To determine the risk factors and outcomes of *Blastocystis* infections, standardized questionnaires concerning demographic data, risk behaviors, and clinical outcomes were included. Diarrhea was defined as a change in the normal pattern of bowel movements and at least three loose stools during a 24-h period, and dysentery was defined as at least one passage of mucous bloody stool in 1 day. The enrolled subjects in each house were asked to collect stool samples and complete standardized questionnaires. Older girls of each house were asked to collect stool samples and complete the questionnaires for some younger girls. The weight and the height of each individual were recorded. To determine their nutritional status, INMU Thai-growth, Ministry of Public Health, Thailand, 1995 was used as standard reference.²⁴

Statistical analysis. The association between potential risk factors and *Blastocystis* infection was assessed by the χ^2 or Fisher's exact test with a 95% confidence interval (CI). Univariate analysis was performed using STATA version 9.2. Odds ratios (ORs) with 95% CIs and *P* values were calculated to compare outcomes among study groups. Logistic regression was performed for multivariate analysis to assess the independent association of risk factors and *Blastocystis* infection.

RESULTS

Prevalence of *Blastocystis* infection. In all, 370 participants from the Home for Girls were enrolled in this study with a response rate of 79.9%. The characteristics of infected participants are shown in Table 1; the median age of the girls was 11.8 (5.5 to 24.9) years. Overall prevalence of *Blastocystis* infection in this community was 31.9% (118 of 370). Of 19 households, *Blastocystis* infection was found in every household with a prevalence ranging from 7.7% to 47.8%. Houses 9, 14, 17, and 19 had a higher *Blastocystis* prevalence compared with the other 15 houses. However, no statistical differences

TABLE 1
Characteristics of the enrolled subjects and the prevalence of *Blastocystis* infection

Characteristic	No. enrolled subjects	No. (%) infected subjects	<i>P</i> value
Age: median (range) years			
11.8 (5.5–24.9)			
House no.			
1	17	4 (23.5)	
2	10	2 (20.0)	
3	17	6 (35.3)	
4	26	6 (23.1)	
5	24	7 (29.2)	
6	20	6 (30.0)	
7	27	9 (33.3)	
8	19	5 (26.3)	
9	23	11 (47.8)	
10	22	8 (36.4)	
11	26	9 (34.6)	
12	13	1 (7.7)	
13	17	7 (41.2)	
14	17	8 (47.1)	
15	20	6 (30.0)	
16	19	2 (10.5)	
17	22	10 (45.5)	
18	18	5 (27.8)	
19	13	6 (46.2)	0.402
Nutrition status			
Normal nutrition status	282	95 (33.7)	
Under nutrition status	33	10 (30.3)	
Over nutrition status	36	10 (27.8)	0.776
Education level			
Kindergarten	3	2 (66.7)	
Primary school	243	71 (29.2)	
Secondary school	60	21 (35.0)	
Others	63	23 (36.5)	0.339
Total	370	118 (31.9)	

were found for *Blastocystis* infection among these households (*P* > 0.05) (Table 1). All *Blastocystis*-infected girls showed no gastrointestinal symptoms.

Using both *in vitro* cultivation and PCR methods, stool specimens collected from six domestic dogs showed negative results for *Blastocystis*. In addition, samples of drinking water collected from every household also gave negative results.

Risk factors of *Blastocystis* infection. Univariate analysis of the risk association for *Blastocystis* infection in this community showed that those with unwashed hands before cooking (OR = 1.8, 95% CI = 1.1–2.8), having a history of contacting pets (OR = 1.9, 95% CI = 1.0–3.5), and with unwashed hands after contacting pets (OR = 2.0, 95% CI = 1.2–3.1) had a higher risk of acquiring *Blastocystis* infection (Table 2). Multivariate analysis showed that the participants who had not washed their hands after contacting pets were 1.8 times at higher risk of acquiring *Blastocystis* infection (95% CI = 1.0–3.0) than those who did after adjusting for age, nutrition status, washing hands before cooking, and contacting pets (Table 2).

Sequence analysis of partial SSU rRNA of *Blastocystis*. Of 118 cultured positive stool samples, PCR amplification was successful for 58 samples (49.2%). Of which, one (1.7%) and 2 (3.5%) positive samples were identified as subtype 2 and subtype 6, respectively. The nucleotide sequences of subtypes 2 and 6 obtained from this study were a 99% match with those previously reported in GenBank (accession no. AB070997 and AB91237, respectively).

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

Characteristic	No. enrolled subjects	No. (%) infected subjects	Crude OR (95% CI)	P value	Adjusted OR (95% CI)	P value
Age: median (range) years						
11.8 (5.5–24.9)	370	118 (31.9)	1.0 (1.0–1.1)	0.581	1.0 (1.0–1.1)	0.479
Nutrition status						
Normal nutrition status	282	95 (33.7)	1		1	
Under nutrition status	33	10 (30.3)	0.9 (0.4–1.9)	0.697	0.8 (0.3–1.9)	0.603
Over nutrition status	36	10 (27.8)	0.8 (0.4–1.6)	0.497	0.9 (0.4–2.0)	0.899
Wash hands before cooking						
Every time	204	54 (26.5)	1		1	
Occasional	154	60 (39.0)	1.8 (1.1–2.8)	0.012*	1.3 (0.8–2.2)	0.293
Contact pets						
No	73	16 (21.9)	1		1	
Yes	281	97 (34.5)	1.9 (1.0–3.5)	0.040*	1.4 (0.7–2.9)	0.304
Wash hands after contact pets						
Yes	228	61 (26.8)	1		1	
No	122	51 (41.8)	2.0 (1.2–3.1)	0.004*	1.8 (1.0–3.0)	0.044*

*Data were adjusted for age, nutrition status, wash hands before cooking, and contact with pets. OR = odds ratio; CI = confidence interval.

Of the remaining samples, 55 samples (94.8%) were subtype 1, which showed 97–99% nucleotide identity to the previously reported sequence (accession no. AF439782). Interestingly, the nucleotide region between positions 616 and 655 of the subtype 1 showed the areas of high diversity (60%) and could be segregated into two different patterns (Figure 1). On the basis of these differences, subtype 1 was further classified as subtype 1/variant 1 and subtype 1/variant 2. The prevalence of these two variants were 63.6% (35 samples) and 16.4% (9 samples), respectively, whereas another 20% (11 samples) were co-infections. The existence of these variants was strongly supported by the posterior probability at 0.93 calculated by Bayesian tree analysis using nucleotide sequences obtained from this study and reference sequences of all 13 subtypes found in *Blastocystis* (Figure 2).

Distribution of *Blastocystis* subtypes in households. Distribution of *Blastocystis* subtypes among girls living in each household is shown in Figure 3 ; in this institute, *Blastocystis* subtype 1/variant 1 was predominant. Of 16 households, the subtype 1/variant 1 was identified in 15 households, whereas subtype 1/variant 2 was found in 5 households (Houses 10, 11, 14, 15, and 17). Co-infection of subtype 1/variant 1 and subtype 1/variant 2 was also found in five households (Houses 3, 9, 10, 13, and 19). Subtype 2 and subtype 6 were found only in Houses 3 and 11, respectively. Unfortunately, none of the positive *Blastocystis* samples in three households, i.e., Houses 2, 16, and 12 were determined for the subtype because of unsuccessful PCR amplification.

DISCUSSION

This study showed that the prevalence of *Blastocystis* infection in the Home for Girls, Bangkok, Thailand was apparently high when compared with the previous com-

munity studies in Thailand (31.9% versus 2.2–18.9%).^{3,11–15} *Blastocystis* can be classified into at least 13 subtypes based on the information of the SSU rRNA gene.^{5,6,19} Among these, subtype 3 is predominant, and usually found in both symptomatic and asymptomatic individuals, except those in Thailand and Brazil.^{12,14,25} Studies from different communities in these two countries showed contrasting results, i.e., that subtype 1 is the most predominant.^{3,14,20} The prevalence of subtype 1 is 1.1 to 26.5 times higher than those of other countries.¹⁰ From our study, the results were consistent with previous findings that subtype 1 was the most common, whereas subtype 3 was not detected. In addition, subtypes 2 and 6 were also identified.

Previous studies have shown the evidence of person-to-person, waterborne, and zoonotic transmission of *Blastocystis* infection^{11–14,20,26,27}; the route of transmission of this organism could be different depending on the study population. In this study, the data concerning all routes of transmission were collected to elucidate the source of infection. The Home for Girls is a close community where most girls live and study in a boarding elementary school located in the area, and thus, the likelihood of getting the infection within the household and also in the community should be very high. Moreover, one-third of the grown-up girls were allowed to study at secondary schools located outside the area where infection of *Blastocystis* could have occurred. In addition, ~3–5 new girls were transferred to stay at the Home for Girls every month and had no parasite screening before moving in. Thus, getting *Blastocystis* infection from outside the community could not be excluded and these could possibly explain the small number of infections caused by subtypes 2 and 6 in some girls.

Person-to-person transmission might be a possible route of *Blastocystis* infection in this community setting because

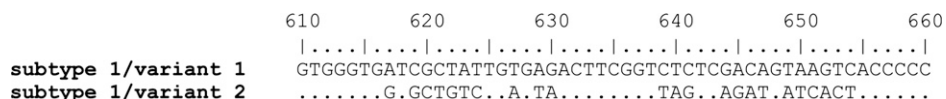


FIGURE 1. Variation patterns of the partial small subunit ribosomal RNA (SSU rRNA) gene of *Blastocystis* subtype 1 at positions between 616 and 655. A dot indicates a nucleotide identical to that of the top sequence.

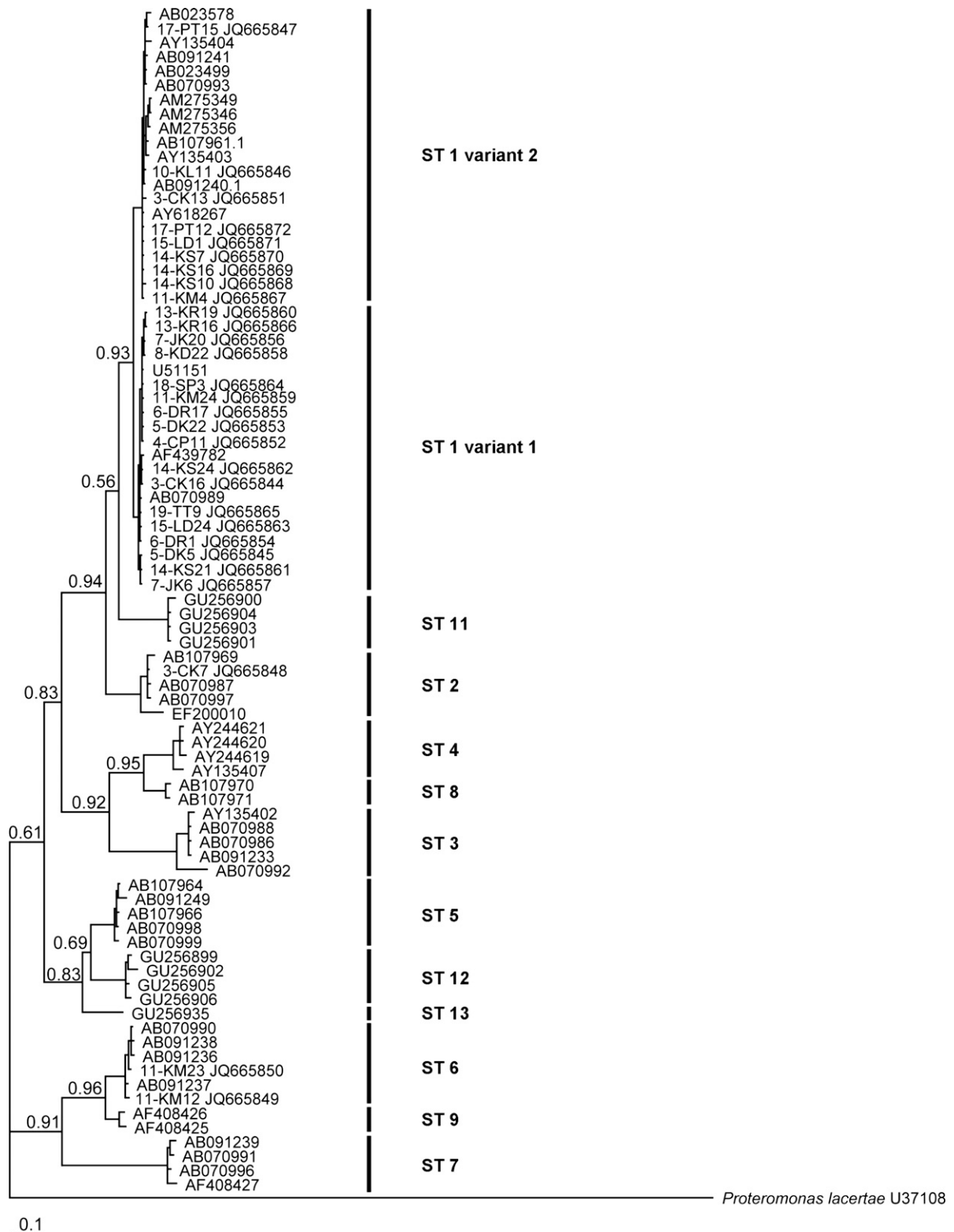


FIGURE 2. The Bayesian phylogenetic tree of *Blastocystis* isolates inferred from partial small subunit ribosomal RNA (SSU rRNA) gene sequences. The representative sequences of *Blastocystis* subtype 1/variant 1, subtype 1/variant 2, subtypes 2 and 6 were used for phylogenetic analysis.

these girls had been sharing all facilities within the same households for months to years. The likelihood of getting *Blastocystis* infection from the others should not be uncommon. Additionally, transferring some girls to other houses

during their stay could spread *Blastocystis* among houses. This assumption could be supported by the distinctly higher prevalence of one subtype over the others. An apparently higher prevalence of the mixed infection of subtypes in

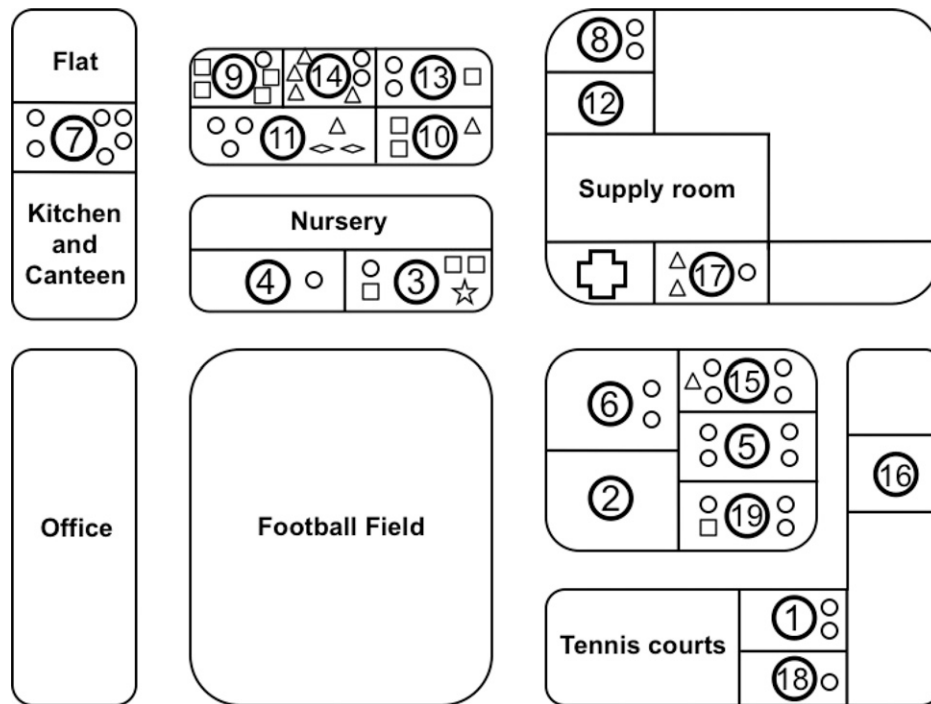


FIGURE 3. Schematic map of the House at Home for Girls and widespread distribution of *Blastocystis* subtype in each household. ○: subtype 1/variant 1, △: subtype 1/variant 2, □: subtype 1/variant 1 mixed with variant 2, ☆: subtype 2, ◊: subtype 6

this community also supported and strengthened the possibility of person-to-person transmission when compared with the prevalence of *Blastocystis* infection in other studies (20% versus 1.1–14.3%).^{28–30}

Zoonotic transmission of *Blastocystis* in this population could be possible. Multivariate analysis showed that the girls who did not wash their hands after contacting pets had a high risk of getting *Blastocystis* infection. Previous studies identified subtype 1 in a wide range of animals and phylogenetic analysis also showed the zoonotic potential of this subtype.^{2–4} However, conclusive evidence of zoonotic transmission could not be revealed in this study because stool samples collected from six dogs showed negative results for *Blastocystis* infection. All stray dogs had been displaced from the community a few weeks before stool samples were collected, thus leaving only six dogs to be included in the study. Additionally, collected stool samples from a number of cats, frequently seen roaming around the area where the girls frequently played, were not successfully done.

Waterborne transmission of *Blastocystis* infection has been indicated in a few studies^{11–14}; a survey of parasitic infections in primary school children of a rural community, central Thailand, showed a high prevalence of *Blastocystis* subtype 1. *Blastocystis* subtype 1 was also found in the drinking water.¹⁴ In this study, samples of drinking water collected from all households including those provided from the canteen, the main kitchen, and the nursing care room showed negative results of *Blastocystis* by both *in vitro* cultivation and PCR. Therefore, evidence did not provide significant support for waterborne transmission in this community setting.

Blastocystis is one of the organisms that have been studied to increase discriminatory power at the subspecies level. In this regard, the technique to assemble the data from different

genes to analyze as a set has been proved to be very promising.³¹ However, the multilocus analysis recently demonstrated by Stensvold and others³² showed insignificant improvement on discriminatory power when compared with the results obtained from a single locus, SSU rRNA. Nevertheless, the results of their study implied that SSU rRNA is a very informative gene and is the target gene of choice for subtyping *Blastocystis*. In fact, the sequence pattern of subtype 1/variant 2 has already been discovered. Nonetheless, this subtype appears as a minority subtype, that to date, only 14 records from 9 reports have been deposited in the GenBank sequence database compared with hundreds of records of subtype 1/variant 1.^{2,3,33–38} As most of the subtype 1/variant 2 isolates were reported in non-community-based studies, the identification of up to 20 isolates in this study emphasized the importance and usefulness of subtype identification in terms of epidemiological studies.

Uneven distribution of each subtype of *Blastocystis* could be explained by having incomplete molecular data because only half of the stool samples were successfully subtyped. Further investigation using a cohort study could provide in-depth information of molecular epidemiology and reveal the potential transmission routes of *Blastocystis* in this community.

In conclusion, polymorphisms of *Blastocystis* subtype 1 were detected in this setting, which could be used as a tool for epidemiological studies. *Blastocystis* subtype 1/variant 1 was predominant among households indicating the infection occurred within the community. Infection with similar subtypes and variants of *Blastocystis* might indicate person-to-person transmission. Risk analysis also provided evidence of zoonotic transmission of *Blastocystis* infection in this community. This information is necessary for setting prevention and control programs for this community.

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