■ ORIGINAL ARTICLE

Subtype Distribution of *Blastocystis* in Thai-Myanmar Border, Thailand

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Abstract: *Blastocystis* sp. is a common zoonotic intestinal protozoa which has been classified into 17 subtypes (STs). A cross-sectional study was conducted to determine the prevalence and subtype distribution of *Blastocystis* in villagers living on the Thai-Myanmar border, where the risk of parasitic infection is high. A total of 207 stool samples were collected and DNA was extracted. PCR and sequencing using primers targeting small-subunit ribosomal RNA (*SSU rRNA*) gene were performed. The prevalence of *Blastocystis* infection was 37.2% (77/207). ST3 (19.8%; 41/207) was the predominant subtype, followed by ST1 (11.6%; 24/207), ST2 (5.3%; 11/207), and ST4 (0.5%; 1/207). A phylogenetic tree was reconstructed using the maximum likelihood (ML) method based on the Hasegawa-Kishino-Yano + G + I model. The percentage of bootstrapped trees in which the associated taxa clustered together was relatively high. Some sequences of *Blastocystis* positive samples (TK18, 39, 46, 71, and 90) were closely related to animals (pig and cattle) indicating zoonotic risks. Therefore, proper health education in parasitic prevention for the villagers should be promoted to improve their personal hygiene. Further longitudinal studies are required to monitor the prevalence of parasitic infections after providing health education and to investigate *Blastocystis* ST in animals living in these villages.

Key words: *Blastocystis*, zoonotic risk, Thai-Myanmar border

INTRODUCTION

Blastocystis is an intestinal protozoa found in humans and animals, as a zoonotic intestinal protozoa [1]. It is one of the most common protozoa identified in human stool samples [2]. The fecal-oral route of transmission is considered to be the mode. Several countries, especially developing countries, show a high infection rate of *Blastocystis* [3]. Based on gene analysis of small-subunit ribosomal RNA (*SSU rRNA*), at least 17 subtypes (STs) have been identified in humans, non-human primates, mammals, and avian hosts [4]. ST1-ST9 were found in humans [5,6], with ST3 being the most common subtype [7,8]. However, *Blastocystis* ST seen in humans were also identified in animals. This suggests that animals may act as reservoirs for *Blastocystis* and may be linked to zoonotic transmission. Therefore, subtyping of this organism is important to identify potential sources and routes of transmission.

Diagnosis of *Blastocystis* is usually based on direct fecal examination in the laboratory. However, this method shows low sensitivity [9] and requires an experienced and skilled microscopist. Culture methods are also available for detecting *Blastocystis* infections. It is a gold standard and more sensitive than direct examination for detection of *Blastocystis* parasites in stool samples [10,11]. However, this method is time-consuming and not routinely performed in many laboratories. PCR, using *SSU rRNA* genes, provides more sensitive and rapid means for *Blastocystis* detection and subtyping [12]. It is becoming more widely used in epidemiological studies for the detection of intestinal parasites in clinical samples.

Along the border of Thailand, local people are at risk of parasitic infections because of poor quality of life and personal hygiene. Furthermore, they have a high chance of close contact with animals due to environmental and cultural conditions, which may cause zoonotic diseases in these areas. In this study,

we aimed to investigate the prevalence of *Blastocystis* infection and the subtype distribution of *Blastocystis* in villagers living on the Thai-Myanmar border.

MATERIALS AND METHODS

Collection of human stool samples

A cross-sectional study was conducted at Mae La sub-district, Tha Song Yang district (Thai-Myanmar border), Tak Province, northern Thailand (Fig. 1). This area is mountainous and difficult to visit or travel through. Therefore, villagers rarely see doctors or public health personnel. Moreover, community socio-economic status and hygiene are quite low. Physical examinations were conducted by a Mobile Clinic from the Hospital for Tropical Diseases in health services during November 2013. Total of 207 human stool samples were collected. The participants were aged from 20 to 70 years. All participants did not complain any gastrointestinal symptoms such as abdominal pain or diarrhea. However, some participants complained myalgia (muscle pain) because of their work in agriculture. The specific instructions for collecting and avoiding contamination of stool samples were clearly explained to all participants. The stool samples were kept in cool conditions during transportation, and then preserved at -20℃ until DNA extraction. The study protocol was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand (MUTM 2014-046-01).

DNA extraction from stool samples

DNA was extracted directly from all fresh stool samples using PSP spin kit (STRATEC Inc., Berlin, Germany), following manufacturer's instructions. Briefly, the stool samples were lysed with lysis buffer under high temperatures (95℃) and the InviAdsorb matrix was used for eliminating undissolved particles (including PCR inhibitors) in the stool samples, followed by proteinase K enzyme to destroy some proteins and increase the purity of DNA. DNA samples were stored at -20℃ until use.

PCR amplification

The presence and characterization of *Blastocystis* STs was carried out using nested PCR on the DNA from collected stool samples. The primer sets and conditions were used according to previously published articles, i.e., external primer set: RD3: GGG ATC CTG ATC CTT CCG CAG GTT CAC CTA C, RD5: GGA AGC TTA TCT GGT TGA TCC TGC CAG TA [13], internal primer set: forward: GGA GGT AGT GAC AAT AAA TC, reverse: ACT AGG AAT TCC TCG TTC ATG [3] and specific for the *SSU rRNA* gene. The PCR products had a length of about 1,100 base pairs (bp) and were detected using 1.5% agarose gel electrophoresis. The DNA in the gel was stained with ethidium bromide and visualized under a transilluminator. PCR amplification for each sample was repeated 3 times.
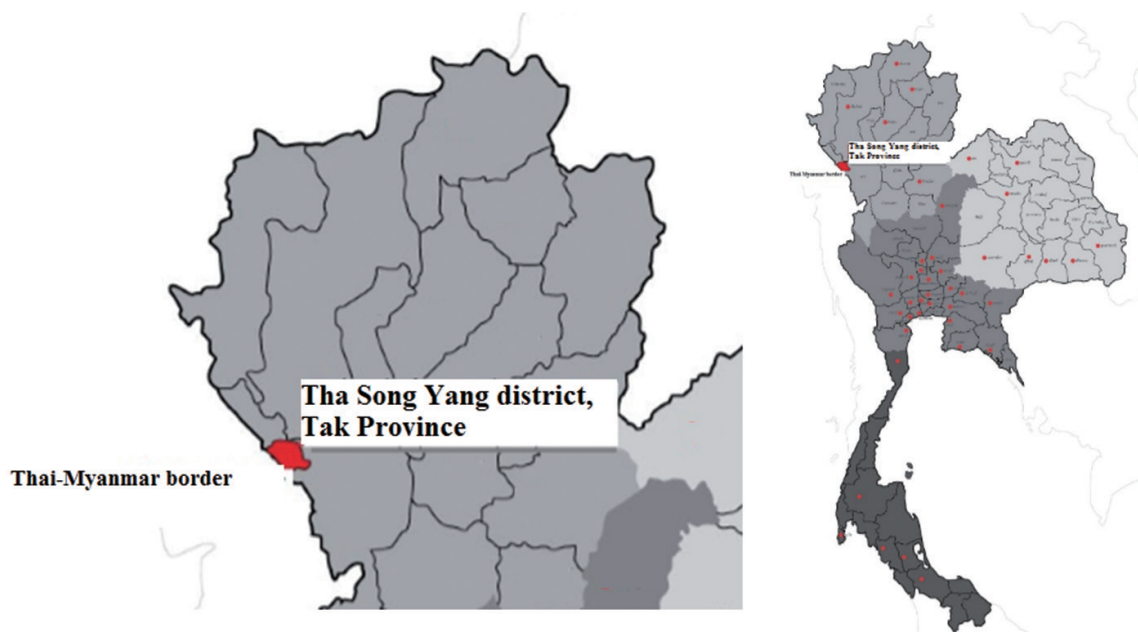

Fig. 1. Mae La sub-district, Tha Song Yang district (Thai-Myanmar border), Tak Province, Northern Thailand.

DNA sequencing and phylogenetic reconstruction

All samples which were tested positive for the *Blastocystis SSU rRNA* gene were sequenced using an ABI 3730 XL sequencer, fluorescent dye-terminator sequencing by Bio Basic Canada Inc. (Ontario, Canada). DNA sequences were read and edited by Bioedit. DNA sequences were compared with published sequences of the *SSU rRNA* gene in 17 STs of *Blastocystis* on available GenBank Database through Blast search (http://blast.ncbi.nlm.nih.gov/Blast.cgi), as shown in Table 1. Multiple alignments were performed by Clustal W [14]. Phylogenetic reconstructions and calculations of genetic divergence (intra- and interspecific divergence) were performed using MEGE 5.2 [15]. Phylogenetic trees were reconstructed using the maximum likelihood (ML) method based on Hasegawa-Kishino-Yano+G (Gamma distribution)+I (Invariant) model (best-fit substitution model). Bootstrap values were calculated by the analysis of 1,000 replicates from the ML analysis. *Proteromonas lacertae* (U37108) closely related to *Blastocystis* was used as an outgroup.

Statistical analysis

Descriptive analysis of percentages was used to explore the positive stool samples and subtype distributions of *Blastocystis*.

RESULTS

Prevalence of *Blastocystis* by nested PCR

The prevalence of *Blastocystis* infection in this study was 37.2 % (77/207) by nested PCR method. Fig. 2 shows the positive PCR results of *Blastocystis*. The PCR products were shown to be about 1,100 base pairs (bp) long.

Subtype characterization of *Blastocystis* in positive stool samples

The subtyping of *Blastocystis* was identified by direct sequencing method. ST1-ST4 were found; ST3 (19.8%; 41/207) was the predominant subtype, followed by ST1 (11.6%; 24/207), ST2 (5.3%; 11/207), and ST4 (0.5%; 1/207), respectively (Table 2). Other STs were not detected.

Phylogenetic reconstruction and genetic divergence of *Blastocystis* isolates

The *SSU rRNA* genes were analyzed for polymorphisms of the DNA sequence, which indicated 139 parsimony informative sites, 46 singleton variable sites, and 185 variable sites from
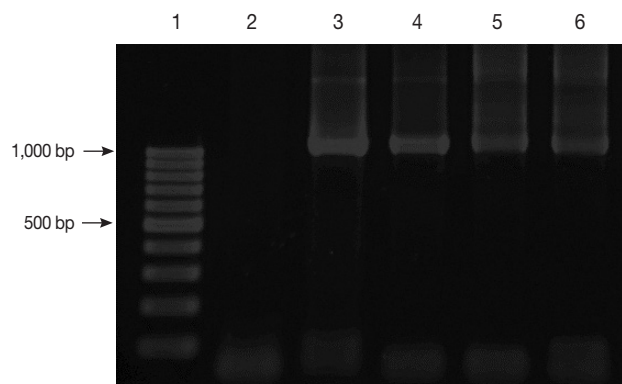
Table 1. Reference sequences of *Blastocystis* sp. for phylogenetic analysis in this study

Subtype	Host	Accession number	Location
1	Pig	AB107961	Japan [28]
	Human	U51151	USA [29]
	Vervet monkey	AB107968	Japan [28]
2	Pig	EU445487	Philippines [30]
	Human	AB070987	Japan [31]
	Pig-tailed macaque	AB107969	Japan [28]
3	Human	AB070992	Japan [31]
	Baboon	HQ909890	Denmark [32]
	Cattle	AB107965	Japan [28]
	Pig	AB107963	Japan [28]
4	Rat	AB071000	Japan [31]
	Human	JN682513	Denmark [33]
	Guinea pig	U51152	USA [29]
5	Pig	AB107964	Japan [28]
	Cattle	AB107966	Japan [28]
	Human	EF468654	China [34]
6	Human	AB091237	Japan [31]
	Chicken	EU445485	Philippines [30]
7	Human	AF408427	Japan [31]
	Duck	AY135412	France [35]
8	Lemur	AB107970	Japan [28]
	Pheasant	AB107971	Japan [28]
9	Human	AF408426	Japan [22]
	Human	KC138681	Denmark [36]
10	Camel	KC148207	UK [4]
11	Elephant	GU256922	Australia [26]
12	Giraffe	GU256902	Australia [26]
13	Mouse deer	KC148209	UK [4]
14	Cattle	KC148205	UK [4]
15	Gibbon	KC148211	UK [4]
16	Red kangaroo	EU427514	Japan [37]
17	Gundi	KC148208	UK [4]

Fig. 2. Gel electrophoresis of PCR amplification. Lane 1, 100 bp DNA marker; Lane 2, negative control; Lane 3, positive control with *Blastocystis*; and Lanes 4-6, positive samples with *Blastocystis*.
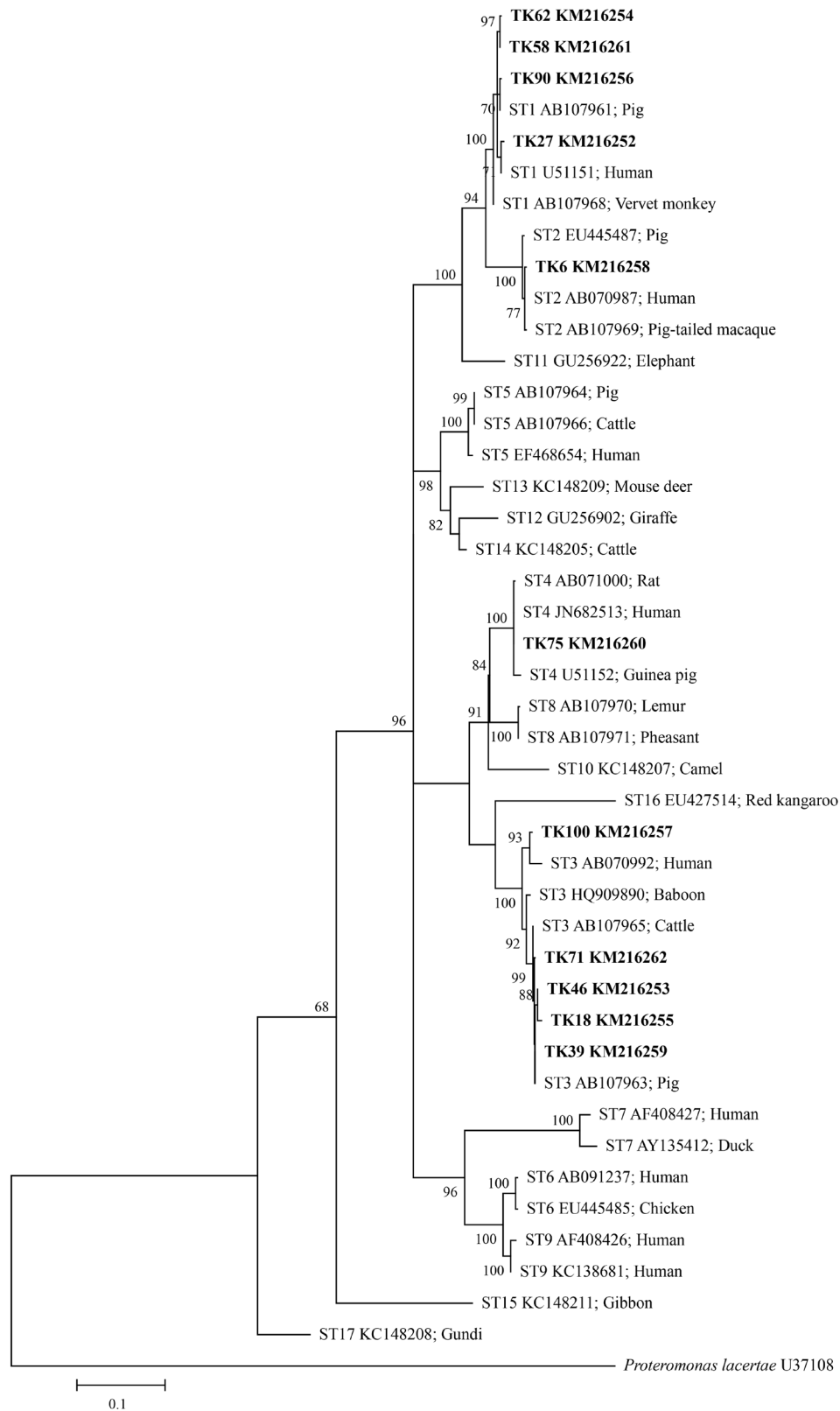
Fig. 3. Maximum-likelihood phylogenetic analysis among *SSU rDNA* sequences of *Blastocystis*. Bold letters are sequences identified in this study.

Table 2. Subtype distribution of *Blastocystis* in this study

Subtype	Number of positive sample (%)
1	24/207 (11.6)
2	11/207 (5.3)
3	41/207 (19.8)
4	1/207 (0.5)
Total	77/207 (37.2)

a total of 1,039 sites. Intraspecific divergence and interspecific divergence ranged from 0.01-2.1% and 3.5-10.8%, respectively. ML tree of *Blastocystis* inferred from *SSU rRNA* gene sequences was shown in Fig. 3. The ML method is based on the Hasegawa-Kishino-Yano+G+I model. The percentage of bootstrapped trees in which the associated taxa clustered together was relatively high. From the ML tree, the *SSU rRNA* gene sequences of *Blastocystis* positive samples compared with 17 reference sequences from available GenBank database showed that TK27, 58, 62, and 90 were classified in ST1, TK6 in ST2, TK18, 39, 46, 71, and 100 in ST3, and TK75 in ST4. Some sequences of *Blastocystis* positive samples (TK18, 39, 46, 71, and 90) were closely related to animals (pig and cattle).

DISCUSSION

In this study, the prevalence of *Blastocystis* infection was 37.2%, a relatively high prevalence. One explanation may be the study area, where poor sanitation and low socio-economic status are common. The prevalence of *Blastocystis* infection varies from country to country as well as within the same country. However, it has been reported that a higher prevalence occurs in developing countries than developed countries [3,16,17]. There are several risk factors for *Blastocystis* infection such as personal and community hygiene, geographic area, culture, and lifestyle of a population. In Thailand, where parasitic infections are still common, *Blastocystis* prevalence has been reported as high as 45% [18-20]. *Blastocystis* is an intestinal protozoan parasite commonly found in humans and a range of animals such as pigs, rodents, chickens, and monkeys. The majority of *Blastocystis* infections are transmitted by the fecal-oral route. The transmission can occur from humans to humans, humans to animals or animals to humans.

To date, the genetic diversity of this parasite has been investigated by *SSU rRNA* gene analysis, showing at least 17 STs [4]. ST1-ST9 have been reported in humans; ST1-ST4 have been shown to be the most common subtypes. *Blastocystis* has been isolated from several kinds of animals (zoonotic STs) belonging to the same subtypes seen in humans. This suggests that animals might act as reservoir hosts for this parasite and facilitate zoonotic transmission. In this study, subtype 3 was the predominant subtype followed by subtypes 1, 2, and 4. The results of subtype distribution are similar to previous studies conducted in many countries such as Singapore, Italy, Bangladesh, and Egypt [3,6,21,22]. Surprisingly, several studies in Thailand reported that ST1 was the common subtype [23-25]. The explanation lies in the difference in PCR protocols between the studies, study areas, and study population, leading to different results of subtype distribution. Previous studies revealed that primers of Böhm-Gloning design did not amplify all *Blastocystis* subtypes, especially subtype 3 [26].

Several methods can be used to detect intestinal parasites in clinical samples. Microscopic examination is one of the choices for detecting *Blastocystis* infection. However, this method shows low sensitivity and specificity, leading to misdiagnosis or underestimation of the true prevalence of *Blastocystis*. Moreover, this parasite shows polymorphic forms and variable size in stool samples, making it difficult to diagnose. The culture method can increase sensitivity and specificity, but it is a labour-intensive and time-consuming method. PCR is an alternative method to detect *Blastocystis* and many other intestinal parasites in epidemiological studies, offering an increase in sensitivity and specificity as well as rapid diagnosis. This method can also characterize the subtypes of *Blastocystis* and reveal the source of infection.

In this study, we used primer sets of nested PCR according to Clark [13] (primary PCR), and Wong et al. [3] (secondary PCR), respectively. The PCR product of the *SSU rRNA* gene was about 1,100 bp. The majority of the primer target contains highly variable regions that allow the construction of a phylogenetic tree. This primer was chosen as if the PCR product size is too short; it is unreliable to perform phylogenetic analysis. When phylogenetic trees were reconstructed based on Hasegawa-Kishino-Yano+G+I model, the results showed that samples TK18, 39, 46, and 71 (ST3) and 90 (ST1) may be related to zoonotic transmission, as shown in Fig. 3. Most villagers sampled work in agriculture. Their traditional culture and lifestyle means they are close to other humans, animals (i.e. pigs, cattle, chickens, and rodents), and the forest, which raises their risk of infection. Many did not identify the dangers associated with contact with infected animals (pathogenic parasites). Their livestock were regularly kept under or near the houses at night.

This increases the chance of parasitic infections due to frequent animal contact, allowing the assumption that zoonotic transmission is more common in rural areas than urban areas. Thathaisong et al. [23] reported that zoonotic transmission might occur in the home for girls in Thailand, as those girls who did not wash their hands after making contact with pets had a higher risk of infection. Local rhesus monkeys were the possible source of *Blastocystis* (ST2) found in children in Kathmandu, Nepal [27].

In conclusion, the zoonotic risks of *Blastocystis* in villagers are considered in this study. The prevalence of *Blastocystis* infection was still relatively high. Therefore, the villagers should be encouraged to improve their personal hygiene, as well as having access to proper health education on parasite prevention. Further longitudinal studies will be required to monitor the prevalence of parasitic infections and impacts of possible health education, and to investigate *Blastocystis* STs in animals living in the villages.

ACKNOWLEDGMENTS

CONFLICT OF INTEREST

We declare that we have no conflict of interest related to this work.

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