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Subtype identification of *Blastocystis* isolated from orphans, Pathum Thani Province, Thailand

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ABSTRACT

Blastocystis is an intestinal protozoan that can be found in humans and many animals, such as reptiles, amphibians, poultry, and insects. *Blastocystis* has a worldwide distribution, and is especially common in developing countries due to poor personal hygiene practices and socioeconomic factors, such as education-level, income, and occupation. The main mode of transmission is by fecal-oral route. To date, seventeen subtypes, ST1-ST17, have been isolated from humans and animals. This study was conducted to determine the prevalence, subtype (ST), and source of *Blastocystis* at an orphanage in Pathum Thani Province, Thailand. Subtypes were identified by direct sequencing and polymerase chain reaction with sequence-tagged site primers (PCR-STS). Eleven bottles of drinking water from each household and canteen, 125 human fecal samples, and 4 dog fecal samples in/around the orphanage were collected. Phylogenetic analysis was used to evaluate subtype characteristics. The prevalence of *Blastocystis* among the Thai male orphans was 51.2% (64/125) by PCR, the highest rate reported in Thailand to date. The predominant subtype was ST3 (79.69%), followed by ST1 (18.75%), and ST2 (1.56%). All drinking water and dog fecal samples were negative for *Blastocystis*. The predominance of ST3 is consistent with most other findings around the world, but not with previous studies done in Thailand. ST3 is considered to be the only original human subtype, and therefore, the potential source of *Blastocystis* in this particular orphanage was human. It is very important to improve personal hygiene among the orphans, to promote better health and quality of life in the orphanage.

Keywords: *Blastocystis*, subtype, orphan

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INTRODUCTION

Blastocystis is an enteric protozoan parasite commonly found in humans and a wide range of animals [1,2], with worldwide distribution [1,3]. Particularly high rates of *Blastocystis* infection are often found in developing countries [4]. In

Thailand, the prevalence of intestinal parasitic infections has been widely investigated, not least *Blastocystis,* which is frequently found in parasitological surveys [5]. The prevalence of *Blastocystis* can be as high as 10-45.2% [6-9].

Different techniques are used to detect *Blastocystis*, including microscopic examination and molecular methods. A diagnosis of *Blastocystis* is usually based on microscopy, but this has low sensitivity and requires expert technicians, which frequently leads to misdiagnosis [10]. Molecular methods provide a powerful and rapid means of *Blastocystis* detection [11,12]. Polymerase chain reaction (PCR) represents an alternative means of detecting and subtyping *Blastocystis* [11,12]. *Blastocystis* isolates are classified into 17 subtypes, based on characterization of the small-subunit ribosomal *RNA* (*SSU rRNA*) gene in humans and many animals [13].

Orphanages are considered a good setting for the surveillance of intestinal protozoa in humans, due to the high prevalence of infection and limited transmission routes. A previous study at a Thai orphanage found that 45.2% of children were infected with *Blastocystis* [9]. The present study investigated the molecular characteristics of *Blastocystis* in an orphanage in Pathum Thani Province, Thailand. The prevalence and source of *Blastocystis* were determined in human and animal feces, and drinking water samples. Subtype analysis was conducted using two methods, direct sequencing and PCR with sequence-tagged site primers (PCR-STS). Phylogenetic analysis was performed to evaluate subtype characteristics.

MATERIALS AND METHODS

Study area and study population

A cross-sectional study was conducted at an orphanage in Pathum Thani Province, Thailand in July 2013.

Collection and DNA extraction of fecal specimens

A total of 129 fecal samples were collected from 125 Thai male orphans and 4 dogs in/around an orphanage. All human participants were aged between 13-20 years. Fecal samples were kept in cool conditions during transportation and preserved at -20°C for DNA extraction. DNA was extracted from the fecal samples using a commercially available DNA extraction kit (PSP Spin Stool Kit, STRATEC Inc. Germany), according to the manufacturer's instructions.

Collection, concentration, and DNA extraction of water samples

Twenty-liter drinking-water samples were collected from each household and canteen in the study area. A total of 11 bottles (9 samples from households, and 2 samples from canteen) of drinking water were concentrated by filtration, via cellulose nitrate filter with a pore size of 0.2 µm (Sartorius Stedim Biotech GmbH, Germany). DNA was extracted from all water concentrates with a QIAamp DNA Mini Kit (Qiagen, GmbH, Hilden, Germany), following the manufacturer's protocol.

PCR amplification

DNA extracted from the fecal and water samples underwent PCR to identify *Blastocystis*, targeting *SSU rRNA*. The PCR protocol was performed using forward primers (5' GGA TTT CGC ACT TGT TCA TC 3') [14] and reverse (5' TGC TTT CGC ACT TGT TCA TC 3'), which can detect *Blastocystis* subtypes [15]. Each 50µl of master mix included 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1 µM of each primer, 1.5 µl BSA (0.1g/10 ml), and 2.5 U Taq (Fermentus, USA). The PCR condition consisted of 1 denaturing cycle at 95°C for 5 min, 35 annealing cycles at 54°C for 30 sec, extension at 72°C for 30 sec, denaturing at 95°C for 30 sec, and final extension at 72°C for 5 min. The PCR products and 100 bp DNA markers were detected by 1.5% agarose gel electrophoresis with 0.5% TAE (Tris-acetate-EDTA) buffer. Gels were stained with ethidium bromide and visualized under a UV transilluminator.

Mixed-subtyping by PCR with STS primers

To date, 7 (ST1-ST7) specific STS primers (SB83, SB155, SB227, SB332, SB340, SB336, and

SB337) have been used for subtype and mixed-subtype identification of *Blastocystis* [16].

Sequence and phylogenetic analysis

DNA sequencing analysis was performed on all PCR-positive *Blastocystis* samples. To confirm positive samples, all nucleotide sequences were subjected to BLAST search in the GenBank database. Table 1 shows the 17 subtypes, with the accession numbers, used in this study. Phylogenetic and molecular evolutionary analyses were carried out using MEGA software version 5.1 [17]. The evolutionary distances between the different isolates were calculated using Kimura 2-parameter method, and phylogenetic trees were constructed using the maximum-likelihood algorithm. Branch reliability was assessed using bootstrap analyses (1,000 replicates).

Statistical analysis

Descriptive analysis was used with percentages to express the positive samples of *Blastocystis*.

Nucleotide sequence accession numbers

The *Blastocystis* sequences in this study were deposited in GenBank, with accession numbers KF285443-KF285450.

Ethical approval

The study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Thailand (CEA # MUTM 2013-032-01). Informed consent/assent forms were obtained from all participants or headworkers prior to fecal sample collection.

Table 1 List of GenBank references used for construction of the phylogenetic tree

Subtype	Host	Location	Accession number	References
1	Human	Japan	AB070989	[38]
	Human	USA	U51151	[39]
	Pig	France	AY135403	[40]
	Human	Thailand	AY618267	[23]
2	Human	Thailand	EF200010	[24]
3	Human	Turkey	AM779042	[41]
	Human	Thailand	AY618258	[23]
	Human	Lebanon	KC294195	[41]
	Human	Denmark	AM712465	[42]
4	Human	Germany	AY244620	[16]
5	Pig	Japan	AB070998	[38]
6	Human	Japan	AB070990	[38]
7	Human	Japan	AF408427	[38]
8	Primate	Japan	AB107970	[43]
9	Human	Japan	AF408426	[16]
10	Cattle	USA	JQ996360	[44]
11	Elephant	Australia	GU256933	[44]
12	Westerngrey kangaroo	Australia	GU256937	[44]
13	Quokka	Australia	GU256935	[44]
14	Cattle	USA	JQ996357	[44]
15	Camel	Libya	KC148210	[13]
16	Red kangaroo	Australia	EU427512	[13]
17	Gundi (comb rat)	Libya	KC148208	[13]

RESULTS

A total of 125 fecal samples were collected from Thai male orphans whose ages ranged from 13 to 20 years. The results showed that 64/125 (51.2%) persons were positive for *Blastocystis* by PCR technique, which was performed three times per sample (Fig 1). Four dog fecal samples and 11 drinking water samples were negative for *Blastocystis*. All positive fecal samples were sequenced and PCR-STS was performed for subtype analysis. In the present study, ST1, ST2, and ST3 subtype infections were observed in fecal samples positive for *Blastocystis*. Among 64 subtypes, ST3 was the predominant (79.69%), followed by ST1 (18.75%), and ST2 (1.56%). There were differences in distribution among subtypes ST1, ST2, and ST3 (Table 2).

Each sequence was aligned with the reference sequence data from previous reports of *Blastocystis* in GenBank. In the phylogenetic analysis, subtype sequences from our study were located in the ST1-ST17 sequence (Fig 2). The phylogenetic results showed two ST1 subgroups: variants 1 and 2. ST3 showed no distinct subgroup. OPTMD isolates 3, 4, and 7 (GenBank accession nos. KF285448-KF285450) showed one base difference among them, and highly similar sequences (99-100%).

DISCUSSION

The key findings of this study are the high prevalence of *Blastocystis*, and the subtype characteristics, including the predominance of ST3. The prevalence of *Blastocystis* was found to be 51.2%, the highest so far reported in Thailand, though similar to a previous report from a Thai orphanage (45.2%) [9]. In Thailand, the prevalence of *Blastocystis* ranges from 0.19%-45.2% [6-9].

Variations in prevalence are due to the different target populations, the areas of investigation, and methods used for detection [18]. *Blastocystis* has been found among Thai soldiers in Chonburi Province (36.9%) and migrant workers in Myanmar (41.5%) [18,19]. The lower prevalence of Blastocystis infection

Table 2 Subtype distribution of *Blastocystis* in this study

Subtype	Number of positive sample (n=64)
1	12/64 (18.75%)
2	1/64 (1.56%)
3	51/64 (79.69%)

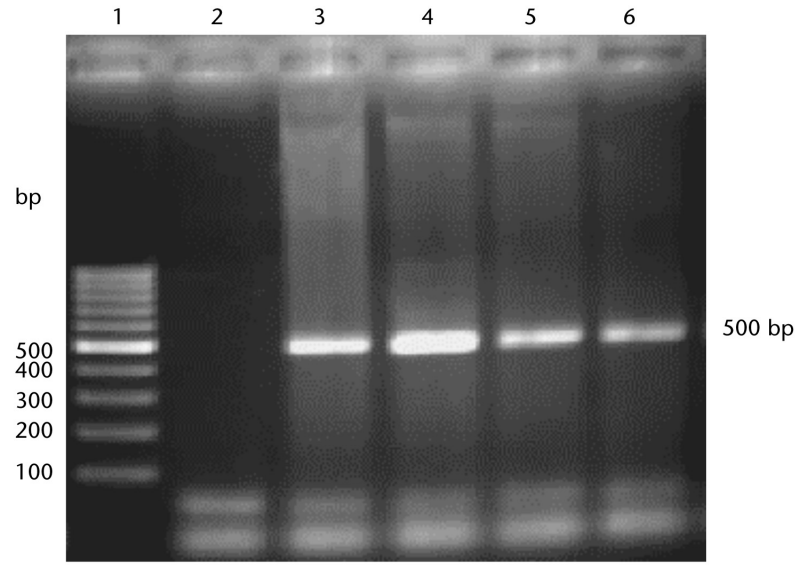

Fig 1 Gel electrophoresis results: Lane 1, DNA marker; Lane 2, negative control; Lane3, positive control; and Lanes 4-6, positive results of *Blastocystis* isolate in this setting.

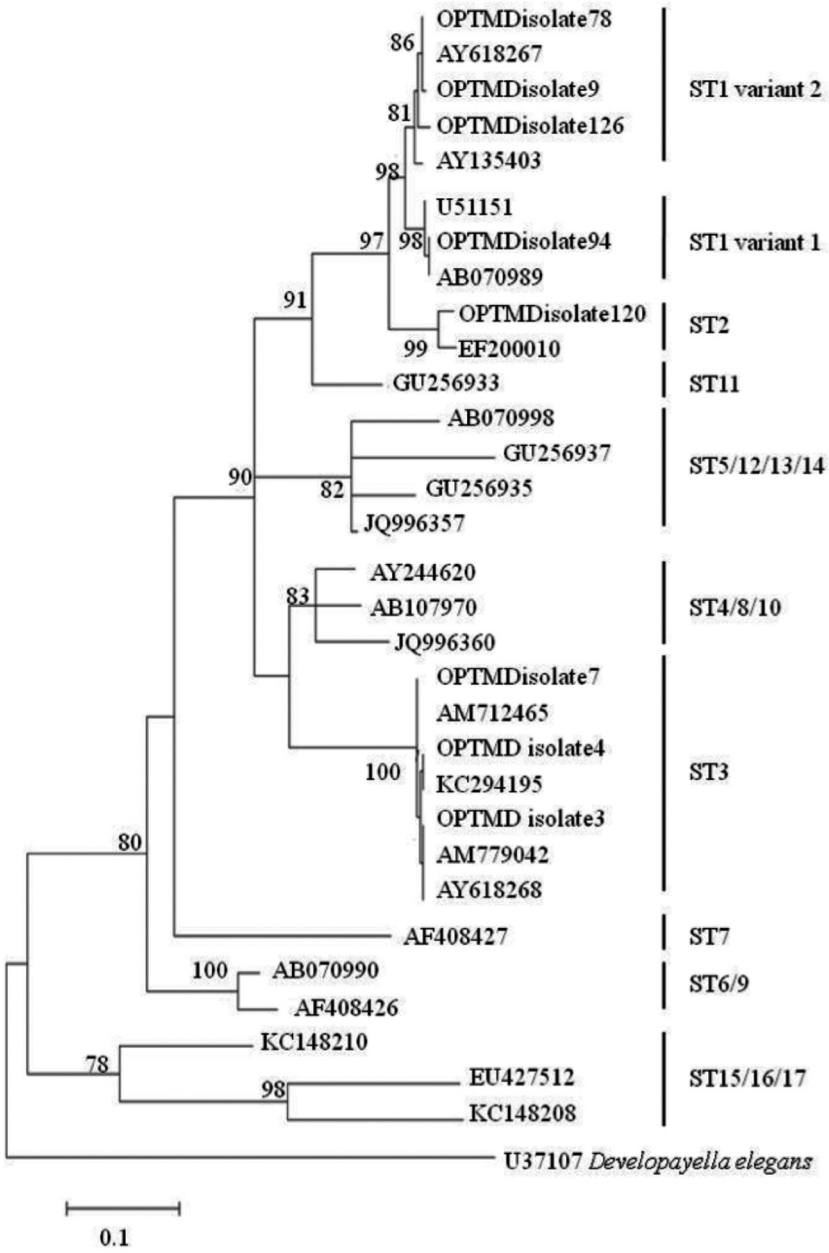

Fig 2 Phylogenetic tree of subtypes of the *SSU rRNA* gene sequences *Blastocystis*, inferred by maximum-likelihood test

found by Viriyavejakul and colleagues [20] in immunocompromised hosts (10.9%) in Thailand was likely due to the use of microscopic methods for detection, which is less sensitive than culture and molecular methods [10,11,19,21]. The culture method is suggested as the gold standard for detection of Blastocystis, but it is not commonly used in many laboratories due to its being labor-intensive and time-consuming [22]. The molecular method was used in the present study, as it provides a powerful and rapid means of *Blastocystis* detection [11,12].

For *Blastocystis* subtype analysis, we used direct sequencing and PCR-STS. The predominant

subtype identified in our study was ST3, consistent with most studies reported all over the world, but not with previous studies in Thailand. In Thailand, ST1 has been reported as the predominant subtype [23,24], except for one investigation in northeastern Thailand [25]. PCR conditions may be behind the discrepancy.

Geographical distribution may also be a cause of inconsistencies in subtype characteristics. At the present time, 17 subtypes of *Blastocystis* have been recognized globally, based on results of molecular diagnosis using the *SSU rRNA* gene, from ST1 to ST17, isolated from humans and a wide range of animals [13]. ST1 to ST9 are found in humans [26,27]. ST3 is the most common subtype found among humans in a number of countries, including Denmark [26], Singapore [28], Egypt [29], Japan, Bangladesh, and Pakistan [16]. However, ST1, ST2, and ST4 have been reported in other regions, such as Jiangxi and Eryuan counties in China, and in Nepal [30-32]. The distribution of *Blastocystis* subtypes in humans varies between countries, and also within various communities in the same country.

Two methods, direct sequencing and subtype-specific primers, have been commonly used to identify *Blastocystis* subtypes. PCR-STS is frequently used to detect mixed infections. However, the primers can reveal only ST1 to ST7. To date, only 17 subtypes have been reported. STS primers cannot indicate all subtypes, which might contribute to underestimating the results [13]. Direct sequencing has the advantage of detecting all subtypes; it is not, however, suitable for analyzing mixed-subtype infections.

Different primer conditions are part of the reason for the different subtype characteristics in Thailand. All previous studies in Thailand that identified ST1 predominance used the same primers to detect *Blastocystis* [24,33]. In the present study, we used two methods for subtype analysis: direct sequencing and PCR-STS. The results of both methods support the predominance of ST3.

Thathaisong and colleagues [33] used the direct culture method from fecal samples, followed by the molecular method to detect *Blastocystis*. We did not perform the culture technique, which may be one reason for the discrepancy. In our study, DNA templates came from direct fecal extraction. In the culture method, some subtypes can overgrow and the technique may miss certain subtypes [34], so the present study used DNA templates from direct fecal extraction.

To find the source of *Blastocystis* in an orphanage, we collected human and animal fecal matter, and drinking water samples that were also reportedly potential sources of *Blastocystis* infection [11,35,36]. Drinking water has been suggested as the source of *Blastocystis* contaminationin both Thailand and Nepal [24,32]. This is due to the fact that *Blastocystis*, especially in its cyst form, is resistant to water treatment, such as chlorination; it can persist in water at room temperature for 2-3 months [37]. Therefore, drinking boiled water is necessary to reduce *Blastocystis* infection [32]. In this study, animal feces and drinking water samples were negative for *Blastocystis*. The potential source of *Blastocystis* in this community, therefore, was human feces. However, more studies of risk factors of *Blastocystis* infection among orphans are needed.

To the best of our knowledge, molecular method employed in this present study may be the reason for a higher prevalence of *Blastocystis* as compared with previous studies in Thailand. ST3, the most prevalent subtype in this study, is a human original subtype and transmitted from human to human. This finding differs from most studies conducted in Thailand that found a predominance of ST1. The difference in subtype distribution may be due to differences in diagnostic methods, sample collection regions, and transmission routes. The mode of transmission in this setting is considered direct human-to-human transmission. In this orphanage, the orphans stayed in the same room and shared facilities, risk factors for human-to-human infection. Therefore, the orphans are encouraged to improve their personal hygiene habits and practices, to reduce *Blastocystis* infection.

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