# PREVALENCE AND DISTRIBUTION OF *BLASTOCYSTIS* INFECTION AMONG CHILDREN FROM FOUR PRIMARY SCHOOLS AFTER WATER TREATMENT IN RURAL THAILAND

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Abstract. Blastocystis infection is a common water-borne intestinal protozoan infection among children in many countries. The aim of this study was to determine the prevalence of *Blastocystis* infection among schoolchildren through two cross-sectional samplings: one before and one after the implementation of water treatment in the study area in order to demonstrate if the water treatment could reduce the prevalence of *Blastocystis* infection among study subjects. The study area was rural, central Thailand. The study subjects were schoolchildren aged 6-15 years attending one of the four study schools in Chachoengsao Province, Thailand. One study sampling was conducted in 2015 before the water treatment program was implemented and one during 2016 after the water treatment program was implemented. Study subjects who were asymptomatic schoolchildren in the study schools were chosen. Exclusion criteria were schoolchildren who had diarrheal symptoms upon stool collection or those who received metronidazole during the 2 weeks prior to sample collection. Each study subject was asked to provide a stool sample which was examined by light microscopy using a wet preparation after short-term *in vitro* cultivation. DNA sequencing and subtype analysis was performed using polymerase chain reaction. A total of 497 subjects were included in the first sampling and 357 in the second sampling. The overall prevalence of Blastocystis infection among study subjects in 2015 was 12.9% (64/497) and in 2016 was 4.8% (17/357); the difference was statistically significant. The most common Blastocystis subtype isolated was subtype I. The overall prevalence of Blastocystis infection at the four study schools decreased significantly after implementing the water treatment program suggesting water treatment is an effective method to reduce intestinal infection due to *Blastocystis* among schoolchildren in the study community.

Keywords: Blastocystis, water treatment, prevalence, children, subtype, Thailand

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#### INTRODUCTION

Blastocystis is a common intestinal protozoa and infection with it is a public health problem among children (Stenzel and Boreham, 1996; Tan, 2008). Most infections are asymptomatic but some may have gastrointestinal symptoms (Boorom et al, 2008; Coyle et al, 2012; Poirier et al, 2012). Modern technology may allow us to determine the public health importance of Blastocystis spp (Andersen and Stensvold, 2016) but there is little data from Thailand about this parasite. Extensive genetic diversity has been found among Blastocystis spp isolated from humans and animals; at least 17 subtypes have been identified based on the small subunit ribosomal RNA (SSU rRNA) gene (Bohm-Gloning et al, 1997; Clark, 1997; Yoshikawa et al, 2004b; Noel et al, 2005; Stensvold et al, 2007; Stensvold et al, 2009; Parkar et al, 2010; Thathaisong et al, 2013). In Thailand, the prevalence of Blastocystis infection has been reported variously to range from 10% to 40% among different populations (Taamasri et al, 2000; Taamasri et al, 2002; Thathaisong et al, 2003; Leelayoova et al, 2004; Leelayoova et al, 2008; Pipatsatitpong et al, 2012; Thathaisong et al, 2013) Several studies have reported an association between blastocystosis and drinking unboiled water indicating waterborne transmission (Taamasri et al, 2000; Leelayoova et al, 2004; Suresh et al, 2005; Li et al, 2007; Leelayoova et al, 2008; Abdulsalam et al, 2012). In addition to waterborne transmission, blastocystosis is more common in rural developing countries (Lee et al, 2012). Blastocystis infection may reflect the poor sanitation. Thus, appropriate water treatment may reduce Blastocystis infection.

In this study, we aimed to determine the efficacy of a water treatment program

for reducing the prevalence of *Blastocystis* infection among schoolchildren from four primary schools in Chachoengsao Province, Thailand by conducting preand post-water treatment analyses of the prevalences of *Blatocystis* infection in the study population. We also aimed to identify the subtype of *Blastocystis* spp to better understand its epidemiology.

### MATERIALS AND METHODS

### Study population and specimen collection

We conducted 2 cross-sectional evaluations of the prevalences of Blastocystis infection among students attending four primary schools in rural Chachoengsao Province, central Thailand: the first prior to the water treatment program (2015) and the second after implementing the water treatment program (2016). The 2 surveys were among different subjects. The aim was to evaluate the prevalences at the school level. Study subjects were students of the 4 study schools aged 6-15 years. Three of the schools and their surrounding communities obtained their water from untreated sources, such as underground or rain water. The forth school had commercial bottled water for the students to drink but the surrounding community used untreated water. Students without gastrointestinal symptoms whose parents gave written informed consent were included in the study. Students with diarrhea or who had received metronidazole in the 2 weeks prior to the study were excluded from the study. Each subject was asked to provide a stool specimen. The stool specimens were examined for Blastocystis by light microscopy using wet preparation after short-term in vitro cultivation in Jones' medium supplemented with 10% horse serum as described previously (Jones, 1946; Leelayoova et al, 2002;

Termmathurapoj et al, 2004).

# DNA extraction and polymerase chain reaction amplification

The DNA was extracted from Blastocystis samples obtained using a tissue Genomic DNA Mini Kit® (Geneaid Biotech, New Taipei, Taiwan) following the manufacturer instructions. The extracted DNA was kept at -20°C until used for polymerase chain reaction (PCR) amplification of the SSU rRNA gene of the Blastocystis. PCR was carried out according to Yoshikawa et al (2000) and yielded 1,790 bp amplicons. The PCR products were electrophoresed on 1% agarose gel and examined for the presence of expected amplicons using ultraviolet (UV) light with the Molecular Imager<sup>®</sup> Gel Doc<sup>TM</sup> XR+ System (BioRad, Hercules, CA).

# DNA sequencing and subtype analysis

The 16 positive PCR products were sequenced by U2Bio (South Korea) and identified using a National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST) search (http://ncbi.nlm.nih.gov /blastn) (Altschul et al, 1990). To generate the phylogenetic tree of Blastocystis, the SSU rRNA gene sequences of Blastocystis obtained in this study were compared with a set of 34 Blastocystis and Proteromonas lacertae sequences as an outgroup retrieved from the GenBank database using MUltiple Sequence Comparison by Log-Expectation (MUSCLE) (Edgar, 2004a; Edgar, 2004b). The Randomized Axelerated Maximum Likelihood (RAxML) tree was constructed using RAxML, version 7.4.2 (Exelixis Lab, Heidelberg, Germany) using GTR matrix (GTR +  $\Gamma$  model) (Stamatakis, 2006) with RAxMLGUI, version 1.3 (Silvestro and Michalak, 2012) where clade stability was evaluated using 1,000 replicates of RAxML bootstrap values. All sequences

obtained in this study were submitted to GenBank under the accession numbers MH465033 to MH465048.

# Statistical analysis

The  $\chi^2$  test for proportions was used to compare differences in the prevalences of Blastocystis infection among schoolchildren at the 4 study schools before and after implementation of water treatment. The association between primary schools and Blatocystis infection was assessed using the  $\chi^2$  test with a 95% confidence interval (CI). Univariate analysis was conducted using R software, version 3.4.4 (Foundation for Statistical Computing, Vienna, Austria). Prevalence ratios (PRs) with 95% CIs and *p*-values were calculated to compare outcomes among primary schoolchildren for the 2 study years, 2015 and 2016.

# **Ethical considerations**

This study was approved by the Ethics Committee of the Royal Thai Army Medical Department. Permission was given by all school principals before the study has conducted. Informed consent was obtained from the parents or guardians of the study subjects prior to being included in the study.

# RESULTS

# Demographic characteristics of study subjects

The demographic data regarding study subjects is shown in Table 1. A total of 497 subjects were included in the study, 40.4% from School A, 38.0% from School D, 11.1% from School B and 10.5% from School C. Fifty-four point three percents of subjects were females. Ninety-two point one percent of the subjects were Buddhist. The mean (range) age of the subjects was 12.5 (7-15) years.

		$T_{-1} = 1 \dots (07)$			
Characteristics	А	В	С	D	10tal, <i>n</i> (%)
Students	201 (40.4)	55 (11.1)	52 (10.5)	189 (38.0)	497 (100)
Gender					
Male	86 (42.8)	30 (54.5)	21 (40.4)	90 (47.6)	227 (45.7)
Female	115 (57.2)	25 (45.5)	31 (59.6)	99 (52.4)	270 (54.3)
Age group in years					
≤11	51 (41.1)	2 (6.9)	5 (19.2)	3 (3.8)	61 (23.6)
12	25 (20.2)	6 (20.7)	3 (11.5)	19 (24.1)	53 (20.5)
13	23 (18.5)	9 (31.0)	4 (15.4)	23 (29.1)	59 (22.9)
≥14	25 (20.2)	12 (41.4)	14 (53.8)	34 (43.0)	85 (32.9)
Age range (years)	7-15	11-14	11-15	10-15	7-15
Mean $\pm$ SD	$11.7 \pm 1.8$	13.1±1.0	13.1±1.3	13.2±1.1	12.5±1.7
Median (IQR)	12 (11-13)	13 (12-14)	14 (13-15)	13 (11-13)	13 (12-14)
Grade					
1	40 (19.9)	5 (9.1)	6 (11.5)	27 (14.3)	78 (15.7)
2	42 (20.9)	7 (12.7)	11 (21.2)	37 (19.6)	97 (19.5)
3	30 (14.9)	7 (12.7)	13 (25.0)	22 (11.6)	72 (14.5)
4	37 (18.4)	8 (14.5)	3 (5.8)	40 (21.2)	88 (17.7)
5	23 (11.4)	17 (30.9)	6 (11.5)	27 (14.3)	73 (14.7)
6	29 (14.4)	11 (20.0)	13 (25.0)	36 (19.0)	89 (17.9)
Religion					
Buddhism	183 (91.0)	54 (100.0)	49 (98.0)	168 (89.4)	454 (92.1)
Christ	17 (8.5)	0	1 (2.0)	20 (10.6)	38 (7.7)
Islam	1 (0.5)	0	0	0	1 (0.2)

Table 1 Demographic characteristics of study subjects by study school.

SD, Standard deviation; IQR, Interquartile range.

#### Prevalence of Blastocystis infection

The prevalences of *Blastocystis* infection by school are shown in Table 2. The prevalences of *Blastocystis* for Schools A, B, C and D prior to water treatment were: 14.9%, 23.6%, 15.4% and 6.9% and after initiation of water treatment were 4.4%, 14.9%, 0% and 3.2%, respectively. The prevalences of *Blastocystis* decreased significantly after water treatment in Schools A (p=0.003) and C (p=0.004) but not for School B (p=0.362) or D (p=0.236).

# Sequence analysis and subtype characterization

On analysis of the SSU rRNA, we found 98-99% identity matching with previously reported samples in the database. The rooted RAxML tree showed 12 previously described clades (subtypes): 1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 12 and 13 that had 97-100% bootstrap values except for subtype 1 which had a <50% bootstrap value, subtype 6 which had a 67% bootstrap value and subtype 13 which had a 69% boot-

Table 2

strap value (Fig 1). We identified 4 subtypes of *Blastocystis* infection in subjects which were subtypes 1, 3, 6 and 7. The most common subtype found in our study was subtype 1 (68.8%), followed by subtype 3 (18.8%), subtype 6 (6.2%) and subtype 7 (6.2%). The subtype of *Blastocystis* identified after water treatment were: School A - type 1, School B - types 1, 3, 6 and 7, School C - none isolated and School D - type 3 (Table 2).

The water treatment types were: reverse osmosis with ultraviolet light disinfection for Schools A and C, water filtration for School B and commercially bottled drinking water for School D.

# DISCUSSION

In our study, prior to initiation of water treatment, the overall prevalence of *Blastocystis* for all 4 study schools was 12.9%. Previous studies from Thailand reported the prevalence of *Blastocystis* to vary from 0.8% to 45.2%. (Waikagul *et al*, 2002; Saksirisampant *et al*, 2003; Yaicharoen *et al*, 2006; Leelayoova *et al*, 2008; Thathaisong *et al*, 2013; Pipatsatitpong *et al*, 2015).

The communities around the study schools usually obtain their drinking water from ground sources or from rain water. Prior to initiation of water treatment, 3 of the 4 study schools had obtained their water from untreated sources. One of the study schools provided commercially prepared bottled water for their students to drink. This school was used as a reference to compare the other 3 study schools with.

	Subtype		-	1, 3, 6, 7	ı	б		
	<i>p</i> -value		0.003	0.362	0.004	0.236		
Prevalences of <i>Blastocystis</i> infection by study school.	After onset water treatment	Prevalence (%) with infection	7 (4.4)	7 (14.9)	0 (0.0)	3 (3.2)	17 (4.8)	
		Number (%) of study subjects	159 (44.5)	47 (13.2)	58 (16.3)	93 (26.0)	357 (100)	
	Before water treatment	Prevalence (%) with infection	30 (14.9)	13 (23.6)	8 (15.4)	13 (6.9)	64 (12.9)	
		Number (%) of study subjects	201 (40.4)	55 (11.1)	52 (10.5)	189 (38.0)	497 (100)	
	- Water treatment		UV-RO <sup>a</sup>	Filter	UV-RO <sup>a</sup>	Commercial drinking bottled water		
		School		В	C	D	Total	T OCT I

<sup>a</sup> UV-RO, Reverse osmosis system with ultraviolet light disinfection



Fig 1-Randomized Axelerated Maximum Likelihood (RAxML) phylogeny of *Blastocystis* based on partial small subunit ribosomal RNA (SSU rRNA) gene sequences. *P. lacertae* served as the outgroup. Bootstrap values (1,000 replicates) are given as percentages above the individual branches. Branches with values <50% are not shown. *Blastocystis* isolated from study subjects are indicated by school bold font. Subtypes (ST) isolated in the study are underlined.

The water treatment in the 3 schools not providing commercially prepared bottled water for their students consisted of UV-RO in 2 schools and filtered water in one school. The filtered water system was chosen because of limited funds. In the school providing commercially prepared bottled water there was no significant different in the prevalences of *Blastocystis* during the 2 surveys since there was no change in water supply. There was a significant reduction in *Blastocystis* prevalences in the 2 schools that used the UV-RO after initiating the water treatment program. However, there was no significant reduction in *Blastocystis* 

prevalence in the school that used a filtered water system after implementation. This suggests the filtered water system is less effective in reducing the prevalence of *Blastocystis* infection among study subjects at the study schools. This also suggests the UV-RO system is effective in reducing *Blastocystis* infection in the study subjects at the study schools.

Based on the SSU rRNA gene, Blastocystis can be classified into at least 17 subtypes (Stensvold et al, 2009; Parkar et al, 2010; Poirier et al, 2012; Thathaisong et al, 2013). The RAxML tree shows similar tree topology to related Baysian analyses (Noel et al, 2005; Leelayoova et al, 2008; Thathaisong et al, 2013) that have classified Blastocystis into 12 subtypes with highly supported bootstrap values in most node divergences. In our study, Blastocystis infections were polyphyletic and consisted of 4 subtypes (subtypes 1, 3, 6 and 7) suggesting mixed subtype infections without restricted distribution in the community. The most common subtype found in our study was subtype 1, followed by subtypes 3, 6, and 7. Other studies from Thailand have also reported subtype 1 being the most common isolate (Thathaisong et al, 2003; Leelayoova et al, 2008; Thathaisong et al, 2013). Leelayoova et al (2008) reported subtype 1 comprised 77.9% of isolates and the only other subtype reported in that study was subtype 2.

A related study has demonstrated similar results that the prevalence of subtype 1 was 77.9% and only two subtypes (1 and 2) were identified based on RFLP in the same community (Leelayoova *et al*, 2008). The finding of additional subtypes 3, 6 and 7 denoted that *Blastocystis* subtypes had a wide diversity and were generally distributed within this community.

Blastocystis subtype 1 has been iden-

tified in humans and a wide range of animals including pigs, horses, monkeys, cattle, rodents, chickens, quail and pheasants, implying potential zoonotic transmission (Thathaisong *et al*, 2003; Yoshikawa *et al*, 2004a; Noel *et al*, 2005). Noël *et al* (2005) reported subtypes 1-7 are not host specific and cross-infective among animal species.

In our study, water treatment with UV-RO significantly reduced the prevalence of *Blastocystis* infection among study subjects but water filtration did not. *Blastocystis* subtype 1 was the most common subtype isolated among study subjects. UV-RO water treatment system should be considered for other schools with a high prevalence of *Blastocystis*. Further studies are needed to determine if similar water treatment systems in the community can further reduce the prevalence of *Blastocystis* in the community.

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