Drinking Water: A Possible Source of Blastocystis spp. Subtype 1 Infection in Schoolchildren of a Rural Community in Central Thailand

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Abstract. In January 2005, a survey of intestinal parasitic infections was performed in a primary school, central Thailand. Of 675 stool samples, Blastocystis was identified with a prevalence of 18.9%. Genetic characterization of Blastocystis showed subtype 1 (77.9%) and subtype 2 (22.1%). Study of the water supply in this school was performed to find the possible sources of Blastocystis. Blastocystis from one water sample was identified as subtype 1, which had a nucleotide sequence of small subunit (SSU) ribosomal RNA (rRNA) gene that was 100% identical to that of Blastocystis infected in schoolchildren. Our information supports the evidence of water-borne transmission in this population.

INTRODUCTION

Blastocystis spp. is one of the most common intestinal protozoa reported in humans globally, with a high prevalence in developing populations. 1 In Thailand, epidemiologic studies of Blastocystis infection showed incidences as high as 10–40% in different populations. 2–4 Several forms of Blastocystis are observed in fecal specimens (i.e., amoeboid, vacuolar, avacuolar, granular, multivacuolar, and cyst). Of these, the vacuolar form is mostly recognized under microscopic examination. Transmission of Blastocystis occurs by the fecal-oral route. It has been postulated that thin-walled cysts are responsible for auto-infectivity in the host, whereas thick-walled cysts effect external transmission. 5 Transmission can be facilitated by the contamination of the environment, food, or water with excreted cysts from the reservoir hosts. Blastocystis cysts, which are 3–6 μm in diameter, remain viable under suitable conditions. Water-borne transmission of blastocystosis is indicated in a few studies. 6–8 Our previous epidemiologic study in army personnel showed a significant association of blastocystosis and drinking unboiled water. 3 It was postulated that infective cysts could possibly be obtained from contaminated drinking water. To date, genotypic characterization using molecular techniques has been used to study the epidemiology of various infections including blastocystosis. These techniques are useful to identify routes of transmission and sources of infections. In this study, a cross-sectional study was conducted to investigate intestinal parasitic infections in schoolchildren of a primary school, Chacherngsao province, central Thailand, during January 2005. Genetic characterization of Blastocystis collected from schoolchildren using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis of the SSU ribosomal RNA (rRNA) gene was performed. Attempts to show the subtype of Blastocystis contaminated in the water supply provided at the school using PCR-RFLP and sequencing analysis of the SSU rRNA gene were also done to support evidence of water-borne transmission because a high prevalence of blastocystosis was noticed. In addition, risk factors associated with blastocystosis in schoolchildren were also analyzed in this study.

MATERIALS AND METHODS

Study population. A cross-sectional study of intestinal parasitic infections was performed in a primary school that consisted of ∼700 children between 6 and 13 years of age, Chacherngsao province, central Thailand, in January 2005. This study was approved by the Ethical Committee of the Royal Thai Army, Medical Department. Informed consents were obtained from parents or guardians before enrollment. A stool specimen from each enrolled student was examined for intestinal parasitic infections under light microscopy by wet preparation and formalin ethyl acetate concentration. Because wet preparation and formalin ethyl acetate concentration was less sensitive for the detection of Blastocystis, cultivation of Blastocystis using Jones medium supplemented with 10% horse serum was performed as previously described. 9–11

Questionnaires. To determine the risk factors and outcomes of parasitic infections, standardized questionnaires concerning demographic data, sanitary behaviors, source and treatment method of drinking water, animal contacts, and a history of gastrointestinal symptoms were included.

DNA extraction of Blastocystis in stool specimens. Positive samples of Blastocystis from culture medium were used for subtype identification. Extraction of genomic DNA of Blastocystis was performed using FTA filter paper as previously described. 12

Water collection and extraction of DNA. To determine the possible source of Blastocystis, samples of the water supply within the school were collected and examined for Blastocystis using PCR. Approximately 3,000 mL of water was collected from five sources in the school area (i.e., water samples from three water reservoirs and two tanks of collected rainwater). DNA extraction was performed using a commercial kit, UltraClean Water DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA). Water samples were enumerated by vacuum filtering through 0.22-μm pore size, and DNA of any organisms present in water was extracted from the trapped filter, following the manufacturer’s instructions. Extracted DNA was kept at −20°C until used for PCR amplification of the SSU rRNA gene of Blastocystis. In addition, PCR for detecting Giardia duodenalis using primers amplifying the glutamate dehydrogenase (gdh) gene was also performed. 13

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RFLP analysis of the Blastocystis SSU rRNA gene. Genotypic characterization of Blastocystis was determined using PCR-RFLP analysis of 1,100 bp of the partial SSU rRNA gene. Extracted DNA was amplified by a pair of primers described by Clark.14 Secondary PCR was performed using a specific pair of primers described by Bohm-Glöning and others.15 The secondary PCR product produced the expected size of 1,100 bp. Digestion of PCR products was performed using three restriction enzymes, HinfI, Rsal, and Alul endonucleases (Gibco BRL, Gaithersburg, MD), separated by 2% agarose gel electrophoresis and visualized under UV light and documented on high-density printing paper using a Uvisave gel documentation system I (Uvitech, Cambridge, UK).

Sequencing and phylogenetic analysis of the SSU rRNA gene of Blastocystis. To confirm the subtypes, nucleotide sequencing of the SSU rRNA gene of Blastocystis from schoolchildren and the water samples was conducted by Bioservice Unit, Bangkok, Thailand. Chromatograms were manually checked and edited using Sequencher version 4.0.5 (Gene Codes Inc., Ann Arbor, MI). Subsequently, nucleotide sequences of the SSU rRNA gene of Blastocystis obtained in this study were multiple aligned with a set of 33 other Blastocystis isolates retrieved from the GenBank database using BioEdit version 7.0.1. Phylogenetic analysis of SSU rRNA was carried out using MrBAYES version 3.1.2.16 Bayesian analyses of the SSU rRNA dataset were performed using the GTR (general time reversible) + I + G (gamma distribution of rates with four rate categories) model of sequence evolution, with base frequencies, the proportion of invariant sites, and the shape parameter α of the I distribution estimated from the data. 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 3.1.2.17 The analysis was carried out with the inclusion of the 16S-like rRNA gene from Proteromonas lacertae (U37108), an organism phylogenetically closely related to Blastocystis as the outgroup.18

Statistical analysis. The association between potential risk factors and Blastocystis infection was assessed by the χ² test with a 95% confidence interval (CI). Univariate analysis was performed using SPSS for Windows version 11.5 (SPSS, Chicago, IL). Odds ratios (ORs) with 95% CIs and P values were calculated to compare outcome among study groups. Logistic regression was performed for multivariate analysis to assess the independent association of risk factors and blastocystosis.

RESULTS

Prevalence of parasitic infections. Of 675 stool samples, 227 (33.6%) schoolchildren were positive for intestinal parasitic infections as shown in Table 1. Approximately 31% had a single parasitic infection, whereas 2.5% had mixed infections. Intestinal protozoa (i.e., Blastocystis [18.9%], G. duodenalis [5.6%], Trichomonas hominis [3.0%], and Entamoeba coli [2.5%]) were predominantly found in this population. Those who were infected with pathogenic parasites were treated with appropriate antipROTOzoal or anthelmintic drugs.

Characterization of schoolchildren with Blastocystis infection. The characteristics of schoolchildren and the prevalence of Blastocystis infection are shown in Table 2. There was no significant difference among sex, age group, family income, parent’s education, number of children per household, history of animal contacts (dogs or cats), type of drinking water at home (treated or untreated water), and gastrointestinal symptoms. However, the prevalence of Blastocystis infection in the children who were positive for G. duodenalis, E. coli, or T. hominis was 4.2 times greater than those who were not.

Genotypic characterization of Blastocystis in stools and water samples. Using a nested PCR amplification of the SSU rRNA gene of Blastocystis, ~ 54% (68/126) of samples were positive for PCR with the expected amplicon at 1,100 bp. Of 68 positive PCR samples, subtype characterization of Blastocystis was performed. The most common banding RFLP patterns of Blastocystis from schoolchildren were identical to subtype 1, with a prevalence of 77.9%. The remaining samples (22.1%) were positive for subtype 2. Figure 1 shows the representative Blastocystis RFLP banding patterns of subtype 1 and subtype 2 produced by HinfI, Rsal, and Alul endonucleases, respectively. There was no mixed subtype infection detected in these PCR-positive samples. Blastocystis DNA was amplified from one water sample collected from a storage tank in the canteen (Figure 2). The other four water samples were negative for Blastocystis and G. duodenalis by specific PCR amplification.

Sequencing and phylogenetic analysis of the SSU rRNA gene of Blastocystis. From the schoolchildren’s specimens, three and one samples of Blastocystis subtype 1 and subtype 2, respectively, were processed for nucleotide sequencing of the SSU rRNA genes. Nucleotide sequences of the SSU rRNA genes of Blastocystis subtype 2 were submitted to GenBank under accession numbers EF200010. The 1,700-bp sequences of Blastocystis subtype 1 from schoolchildren and water samples and 1,022-bp sequences of Blastocystis subtype 2 were multiple aligned with a set of 33 other Blastocystis isolates retrieved from the GenBank database. An inferred phylogenetic tree of SSU rRNA sequences of Blastocystis from the water samples and schoolchildren with 33 sequences of Blastocystis in the GenBank database and the outgroup, Proteromonas lacertae, was constructed to examine the genetic relationships. The rooted maximum-likelihood tree of Blastocystis sequence alignment identified nine clades of subtypes 1–9 with strong support by Bayesian posterior probabilities (BPs) of 100% for each clade (Figure 3). Blastocystis identified from the schoolchildren and the water sample were clustered with subtype 1 and showed 100% identity with our previously report Blastocystis (accession number AF439782).
In addition, another subtype of *Blastocystis* isolate (accession number EF200010) identified from the schoolchildren formed a monophyletic group sharing within the clade of *Blastocystis* subtype 2 with a BP value of 100%.

**Risk factors of Blastocystis infection.** Using univariate and multivariate analysis, sex, age, family income, parent’s education, number of children per household, history of animal contacts, and type and treatment method of drinking water at home were not significant risks of *Blastocystis* infection in these children. Those who had other intestinal protozoal infections, including *G. duodenalis*, *E. coli*, and *T. hominis*, had 11.3 times greater risk of getting *Blastocystis* infection (95% CI, 5.0–25.5; *P* < 0.001). When only *Blastocystis* subtype 1 was analyzed, this risk still remained, with an OR of 5.0 (95% CI, 2.1–12.0; *P* < 0.001). However, there was no significant association between this risk and *Blastocystis* subtype 2 (OR = 1.8; 95% CI, 0.2–14.6; *P* = 0.448).

**DISCUSSION**

Studies of *Blastocystis* infection in Thai children showed that the prevalence ranged from 0.8% to 45.2% depending on the study population and the detection methods.19–22 The studies that used *in vitro* cultivation for the detection of *Blastocystis* infection showed rather high prevalences.20,22 In this study, the highest prevalence of *Blastocystis* was found among intestinal parasitic infections. To understand the transmission of *Blastocystis* in this population, subtyping of *Blastocystis* from schoolchildren and water sources was performed. PCR-RFLP of the SSU rRNA gene has been widely used to characterize *Blastocystis*, which could differentiate the organism into clades, groups or subtypes, subgroups, ribodemes, and

**FIGURE 1.** PCR-RFLP patterns of *Blastocystis* SSU rRNA gene from stool specimens of children. **A**, Subtype 1. **B**, Subtype 2. **M**, molecular marker (100-bp ladder). Lanes 1–3 show restriction enzyme profiles of 1,100 bp of the SSU rRNA gene digested by *Hinfl*, *RsaI*, and *AluI*, respectively.

**FIGURE 2.** PCR product at 1,100 bp of the SSU rRNA gene of *Blastocystis* (Lane 6) detected in one water sample in a storage tank. **M**, molecular marker (100-bp ladder). Lanes 1–5 show negative amplification of other water samples.

**TABLE 2**

Characteristics of the enrolled primary schoolchildren and prevalence of blastocystosis

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Number (%)</th>
<th>Prevalence of blastocystosis (%)</th>
<th><em>P</em> value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>336 (51.2)</td>
<td>65 (19.3)</td>
<td>0.614</td>
</tr>
<tr>
<td>Female</td>
<td>320 (48.8)</td>
<td>57 (17.8)</td>
<td></td>
</tr>
<tr>
<td><strong>Age group (years)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 9</td>
<td>235 (35.9)</td>
<td>42 (17.9)</td>
<td>0.711</td>
</tr>
<tr>
<td>≥ 9</td>
<td>420 (64.1)</td>
<td>80 (19.1)</td>
<td></td>
</tr>
<tr>
<td><strong>Parent’s primary education</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>82 (12.5)</td>
<td>19 (23.2)</td>
<td>0.255</td>
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<tr>
<td>Incomplete</td>
<td>574 (87.5)</td>
<td>103 (17.9)</td>
<td></td>
</tr>
<tr>
<td><strong>Number of children/household</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 3</td>
<td>118 (18)</td>
<td>24 (20.3)</td>
<td>0.591</td>
</tr>
<tr>
<td>&gt; 3</td>
<td>538 (82)</td>
<td>98 (18.2)</td>
<td></td>
</tr>
<tr>
<td><strong>Frequency of dog contact, three times or more/wk</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>269 (41)</td>
<td>55 (20.4)</td>
<td>0.310</td>
</tr>
<tr>
<td>No</td>
<td>387 (59)</td>
<td>67 (17.3)</td>
<td></td>
</tr>
<tr>
<td><strong>Frequency of cat contact, three or more times/wk</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>189 (28.8)</td>
<td>42 (22.2)</td>
<td>0.129</td>
</tr>
<tr>
<td>No</td>
<td>467 (71.2)</td>
<td>80 (17.1)</td>
<td></td>
</tr>
<tr>
<td><strong>Drinking water (at home)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated water</td>
<td>527 (80.3)</td>
<td>99 (18.8)</td>
<td>0.802</td>
</tr>
<tr>
<td>Treated water (boiled or filtered)</td>
<td>129 (19.7)</td>
<td>23 (17.8)</td>
<td></td>
</tr>
<tr>
<td><strong>Co-infection with other protozoan parasites</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>29 (4.4)</td>
<td>20 (68.9)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>No</td>
<td>627 (95.6)</td>
<td>102 (16.3)</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>656 (100)</td>
<td>122 (18.6)</td>
<td></td>
</tr>
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</table>
clusters depending on the authors’ criteria. However, because the Blastocystis SSU rRNA gene exhibits a high degree of genetic diversity, PCR-RFLP results may be very confusing or difficult to interpret. Thus, a consensus of Blastocystis subtypes has been recently provided based on the analysis of published SSU rRNA gene sequences.\textsuperscript{23} Distribution of Blastocystis subtypes is geographically different; most studies showed that subtype 3 was most frequently observed in humans (61%).\textsuperscript{24} Our previous study showed that subtype 1 was the most predominant in Thailand, followed by subtypes 3 and 7.\textsuperscript{25} A study in a different area of Thailand identified subtypes 5 and 6.\textsuperscript{26} In this study, the most common subtype identified in the schoolchildren was subtype 1, followed by subtype 2. The absence of Blastocystis subtype 3 in this population might be simply explained by a different geographic distribution. Interestingly, Blastocystis subtype 3 is probably the only subtype of human origin. Recent studies conducted in urbanized cities such as Japan, Denmark, and Singapore where zoonotic transmission might be limited found that Blastocystis subtype 3 was predominant.\textsuperscript{27–30} In contrast to a study from Jiangxi, China, subtype 1, previously identified in various kinds of animals, was predominantly found in humans.\textsuperscript{31} This study was conducted in the rural community of central Thailand where zoonotic transmission of intestinal protozoal infection might occur.\textsuperscript{32} On the other hand, the absence of Blastocystis subtype 3 could be caused by the limited sensitivity of the detection method for Blastocystis subtype 3.\textsuperscript{28} Because nearly one half of the samples

**Figure 3.** Maximum-likelihood phylogeny of Blastocystis isolates inferred from SSU rRNA gene sequences. The 1,700-bp sequences of Blastocystis subtype 1 from schoolchildren and water sample and 1,022-bp sequences of Blastocystis subtype 2 were used for the phylogenetic analysis.
gave a negative PCR result, we cannot rule out the existence of other subtypes. It has been suggested that the use of multiple primer pairs is very useful to make sure that all subtypes have been identified.28

Pathogenicity of Blastocystis is still not clear because of conflicting reports on clinical symptoms caused by Blastocystis infection. Most studies showed that most cases of blastocystosis were asymptomatic; however, symptomatic cases with non-specific gastrointestinal symptoms, including irritable bowel syndrome, were also reported.33,34 It has been shown that the amoeboid form may be responsible for gastrointestinal symptoms because it presented predominantly in symptomatic patients.35 Phylogenetic analysis of the SSU rRNA gene using arbitrarily primed PCR (AP-PCR) also showed distinct clades of Blastocystis between those of symptomatic and asymptomatic patients.36 Thus, distinct subtypes of Blastocystis may have different role in its pathogenicity. In this study, no specific gastrointestinal symptoms were found in these Blastocystis-infected children.

Blastocystis has been detected in humans and wide varieties of animal hosts.18,25,26,37 The evidence suggesting the possibility of blastocystosis as a zoonosis was from genotypic characterization of the organism in humans compared with those found in animals.25,26 Interestingly, some subtypes can be identified in both humans and animals, which indicates a zoonotic potential of these subtypes. For example, subtype 1 could be identified both in humans and a very wide range of animals (i.e., pigs, horses, monkeys, cattle, rodents, chickens, quails, and pheasants).25,37,38 In natural settings, subtype 5 was also identified both in humans and dogs from the same village in Thailand.26 A recent study using animal models showed that several human subtypes of Blastocystis could infect chickens and/or rats, confirming the zoonotic potential of these subtypes. For example, subtype 1 could be identified both in humans and animals, which indicates a zoonotic potential of these subtypes. For example, subtype 1 could be identified both in humans and a very wide range of animals (i.e., pigs, horses, monkeys, cattle, rodents, chickens, quails, and pheasants).25,37,38 In natural settings, subtype 5 was also identified both in humans and dogs from the same village in Thailand.26

In contrast, waterborne transmission of blastocystosis has been suggested by a number of epidemiologic studies. Our studies indicated that those who consumed untreated water had higher risk of getting Blastocystis infection.3,6 Recently, it was identified that infections caused by subtype 3 were associated with drinking unboiled water.7 Water-borne transmission of blastocystosis is not unexpected because Blastocystis cysts can survive in different types of water. A study by Suress and others8 showed that viable Blastocystis cysts were detected in sewage samples. In addition, it has been shown that cysts of Blastocystis could survive in chlorinated water at standard concentrations.41 Drinking water used by schoolchildren in this school was mainly from rainwater that was stored in two tanks. No further treatment of drinking water by filtration or boiling was done. Blastocystis was identified by PCR but not conventional microscopy or in vitro cultivation. We detected Blastocystis subtype 1 in a water sample from one of these storage tanks. The nucleotide sequence of the SSU rRNA gene showed 100% identity to those of subtype 1 found in stool specimens of schoolchildren. Thus, it can be postulated that contaminated rainwater might be the source of Blastocystis infection in this school. Contamination of the rainwater could occur along the roof, pipe, or in the storage tank. Although we did not examine the possible sources of contaminants, it was likely that contaminations could be from droppings of some animals. As mentioned above, a wide range of animals could be a reservoir of Blastocystis, especially subtype 1. Unfortunately, we did not have the epidemiologic data to support the association of drinking water at school and blastocystosis because we only included the information of water at home and not at school in our questionnaire. In contrast, our epidemiologic data showed no significant association between the prevalence of Blastocystis infection with types of drinking water at home. As a result, Blastocystis infection was less likely to occur at home. Thus far, techniques to detect Blastocystis in water samples have not been standardized. The method used in this study was easy to perform and allowed using less amount of water samples (3,000 mL). Thus, this technique could be potentially applicable to detect Blastocystis. Further study for improving and validating the protocols for the detection and genotypic characterization of Blastocystis in water should be performed.

In this study, co-infections of G. duodenalis and T. hominis were significantly associated with Blastocystis infection, particularly subtype 1. It has been well recognized that water is the main route of transmission of G. duodenalis.42 Although DNA of G. duodenalis could not be detected by PCR amplification in these water samples, this epidemiologic data possibly support evidence of water-borne transmission of these intestinal protozoa. This emphasizes the importance of providing safe drinking water for preventing these protozoa infections.

In conclusion, we describe the possibility of waterborne transmission of Blastocystis using molecular techniques. Subtype 1, which was shown in stool specimens of schoolchildren and drinking water in this school, supports the evidence of waterborne transmission of Blastocystis. This study draws attention to public health policy for development control program to reduce the morbidity of intestinal protozoa infections, especially waterborne zoonotic disease such as blastocystosis in schoolchildren.

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