RESEARCH NOTE

GENETIC SUBTYPES OF *BLASTOCYSTIS* ISOLATED FROM THAI HOSPITALIZED PATIENTS IN NORTHEASTERN THAILAND

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Abstract. *Blastocystis* sp is probably the most common intestinal protozoan of humans. This taxon is known to include more than 17 subtypes, some of which likely cause human disease. We investigated the distribution of *Blastocystis* subtypes in Thai patients admitted for a variety of conditions at a hospital in northeastern Thailand. Fresh fecal samples, positive for *Blastocystis* by microscopy, were individually cultured in Jones' medium (n = 20) and each sample was used for amplification and sequencing a fragment of 18S rDNA. BLAST search and phylogenetic analysis demonstrated that *Blastocystis* subtypes ST1 (20%), ST3 (60%), ST6 (10%) and ST7 (10%) were present. No clear link between gastro-intestinal symptoms and any particular subtype of *Blastocystis* was apparent. Thus, there is a need to extend the work to evaluate clinical signs and subtypes in a larger cohort of patients.

Keywords: *Blastocystis* subtype, PCR, phylogenetic tree, 18S rDNA sequence

INTRODUCTION

Blastocystis sp is a food-borne protozoan found in human feces worldwide (Noël *et al*, 2005). The prevalence of blastocystosis varies among countries, being as high as 10% and 50% in developed and developing countries, respectively (Tan *et al*, 2002; Thathaisong *et al*, 2003; Boorom *et al*, 2008; Eroglu *et al*, 2009; Laodim *et al*, 2012). The pathogenicity of *Blastocystis* sp is of some debate, from a harmless commensal protozoan (Stenzel and Boreham,

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1996) to the cause of skin rash, abdominal pain, constipation, diarrhea, alternating diarrhea and constipation, irritable bowel syndrome, vomiting, and fatigue (Qadri *et al*, 1989; Boorom *et al*, 2008; Verma and Delfanian, 2013).

At least 17 *Blastocystis* subtypes (STs) can be differentiated by using barcode sequences of the small-subunit rRNA gene by PCR and sequencing (Alfellani *et al*, 2013). STs 1-9 have been found in humans, of which STs 1-4 are the most common (Stensvold *et al*, 2009; Parkar *et al*, 2010; Alfellani *et al*, 2013). STs 4-8 can also be found in monkeys, non-human primates, birds, pigs and rodents (Parkar *et al*, 2010). Dogruman-Al *et al* (2008) reported that ST3 is the most common genotype in both symptomatic and asymptomatic patients while ST2 is a nonpathogenic genotype in Turkey. Similarly, Souppart *et al* (2010) found *Blastocystis* ST3 as the common subtype in symptomatic Egyptians. On the other hand, Eroglu *et al* (2009) reported that ST3 is the dominant genotype in asymptomatic patients and *Blastocystis* ST1 in symptomatic patients in southern Turkey. *Blastocystis* ST1 is significantly more prevalent among symptomatic patients in Lebanon (El Safadi *et al*, 2013). Poirier *et al* (2012) suggested that *Blastocystis* ST4 and ST7 cause irritable bowel syndrome in humans, but Fouad *et al* (2011) found that *Blastocystis* ST3 and ST4 in both asymptomatic and irritable bowel syndrome patients in Egypt. Jantermtor *et al* (2013) reported ST3 to be the most common genotype, followed by ST1 with occasionally ST6 and ST7 in patients from hospitals in northeastern Thailand, but no clear link between the *Blastocystis* STs and clinical parameters was demonstrated.

As the pathogenicity of different *Blastocystis* STs remains unclear and differs depending on the geographical location even in the same country, in this study the identities of *Blastocystis* isolates from hospitalized patients in northeastern Thailand were investigated.

MATERIALS AND METHODS

Patients

Twenty fecal samples were collected between November 2011 and March 2012 from patients diagnosed with a variety of symptoms admitted to Srinagarind Hospital, Khon Kaen, northeastern Thailand. The stool samples were examined as part of investigations into other diseases. Thirteen patients were male and seven female, with age ranging from 9 to 78 years (Table 1). Five patients had various gastrointestinal (GI) symptoms, diarrhea (n = 3) and abdominal pain (n = 2) (Table 1). Relevant data, such as demographic data, clinical features and reason for hospitalization, were obtained for each patient. This study was approved by the Khon Kaen University Ethics Committee for Human Research (HE531382).

Detection and isolation of *Blastocystis* from fecal samples

Fecal specimens were initially examined using a standard formalin ethyl acetate concentration technique (Elkins *et al*, 1986) and cultured in Jones' medium (Jones, 1946) supplemented with 5% horse serum (Life Technologies, Carlsbad, CA). In brief, 1 g of feces was added to 4 ml of Jones' medium and incubated at 37°C for 48 hours and the culture was identified using a light microscope. The spherical cells of vacuolar and granular forms, in size ranging from 2 μm to 200 μm were identified as *B. hominis* (Fig 1). *Blastocystis* suspension was transferred to fresh Jones' medium supplement with 5% horse serum and cultured at 37°C for a further 24-48 hours. After one or two subcultures,

Table 1
Blastocystis isolates subtypes and patients' biodata.

No.	Gender	Age (years)	Reason for hospitalization	Diarrhea	Gastro-intestinal symptoms	*Blastocystis* subtype
1	Female	42	Ovarian tumor	+	+	7
2	Female	41	Systemic lupus erythematosus	-	-	1
3	Female	71	Hypertension	-	-	3
4	Male	78	Diabetes mellitus	-	-	1
5	Female	76	Cerebral cryptococcosis, corneal ulcer	-	-	1
6	Male	44	non-Hodgkin lymphoma	-	+	3
7	Male	38	(HIV) infection	-	-	6
8	Male	71	Chronic renal failure	+	+	6
9	Male	53	Acute nasopharyngitis	-	-	3
10	Male	73	Inguinal hernia	-	-	7
11	Male	73	Corneal ulcer	-	-	1
12	Male	52	Diffuse large B cell lymphoma	-	-	3
13	Male	53	Hyperlipidemia	-	-	3
14	Male	73	Congestive heart failure	-	-	3
15	Male	27	Acute leukemia	-	-	3
16	Female	25	Nephrotic syndrome	+	+	3
17	Female	9	Systemic lupus erythematosus	-	-	3
18	Female	61	(Hepatitis B virus) infection	-	+	3
19	Male	71	Cholangiocarcinoma	-	-	3
20	Male	66	Lung cancer	-	-	3

Blastocystis suspension was centrifuged at 2,500*g* for 20 minutes. The pellet was re-suspended in 1 ml of Jones' medium and centrifuged at 12,000*g* for 10 minutes, and the pellet taken up in an equal volume of 95% ethyl alcohol and stored at -70°C until used for DNA extraction.

PCR determination of *Blastocystis* ST

DNA was extracted from each *Blastocystis* suspension (200 µl) using QIAamp® DNA stool mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and stored in 100 µl of distilled water at -20°C until used. PCR was carried out in a 25 µl volume containing PCR buffer (10 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$), 200 µM dNTPs, 0.2 µM of each primer [to amplify a 479 bp fragment of *Blastocystis* 18S rDNA containing a variable region that allows subtyping (Santín *et al*, 2011)], 0.625 U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) and 2 µl of DNA solution. Thermocycling (carried out in GeneAmp® PCR System 9700; Applied Biosystems, Singapore) conditions were as follows: 95°C for 4 minutes; 35 cycles of 95°C for 30 seconds, 47°C for 30 seconds, and 72°C for 30 seconds; with a final heating at 72°C for 5 minutes. Amplicon was electrophoresed in 1.5% agarose gel, visualized by ethidium bromide staining, excised and purified by the standard method (Vogelstein and Gillespie, 1979) for DNA sequencing, performed using MegaBACE

1000 DNA Analysis System (GE Healthcare, Piscataway, NJ). Sequence were analyzed using BLAST-N search (National Center for Biotechnology Information, Bethesda, MD) and deposited in GenBank (Fig 2). Published 18S rDNA sequences from all *Blastocystis* STs were aligned with these sequences (alignment length of 439 bp trimmed to the length of the shortest sequence) using ClustalW multiple alignment options (Thompson *et al*, 1994) implemented in BioEdit version 7.1.3.0 (Hall, 1999). A maximum likelihood tree was constructed using MEGA 5.2 (Tamura *et al*, 2011) and the best-fit substitution model determined usingTamura 3-parameter (T92+G) model with uniform rates among sites, but assuming a proportion (0.23) of invariant sites.

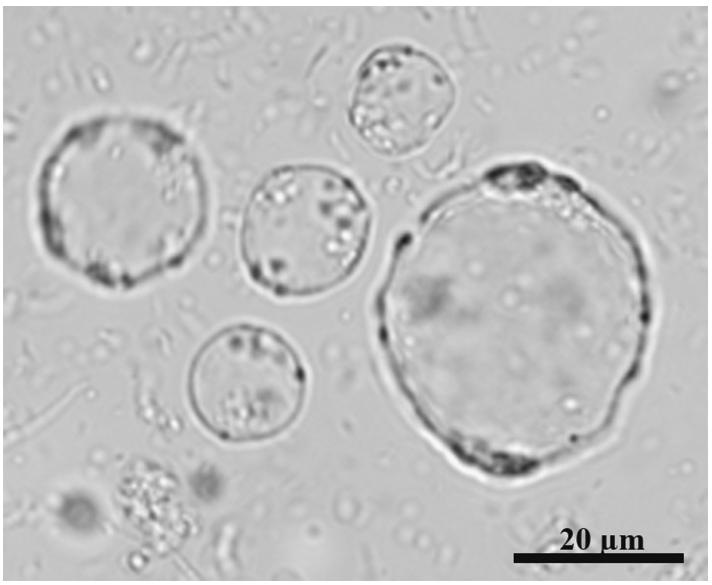

Fig 1–*Blastocystis* sp (vacuolar form) from stool of a patient attending Srinagarind Hospital, Khon Kaen, Thailand (light microscope; x 400 magnification).

RESULTS

An amplicon of about 500 bp was successfully generated from all 20 specimens (data not shown). The partial 18S rDNA sequences were distributed among known *Blastocystis* subtypes as follows: ST1 (20%, 4/20), ST3 (60%, 12/20), ST6 (10%, 2/20) and ST7 (10%, 2/20) (Table 1, Fig 2).

The five patients with GI symptom (3 with diarrhea) had infection of *Blastocystis* subtype ST3 (n = 3), ST6 (n = 1) and ST7 (n = 1), whereas the remaining 15 patients without GI symptom harbored *Blastocystis* ST3 (n = 9), ST1 (n = 4), ST6 (n = 1) and ST7 (n = 1) (Table 1).

DISCUSSION

Given the controversy concerning the possible pathogenicity of *Blastocystis* sp, identification of ST present in patients is important for clarifying any epidemiological association between particular STs and clinical features of the infection (Stensvold *et al*, 2007; Stensvold, 2012). From specimens isolated from 20 Thai patients with *Blastocystis* sp in stool based on microscopic examination, using partial sequences of 18S rDNA it was found that ST3 (60%) was the most common subtype both in symptomatic and asymptomatic GI patients, as also noted by others (Souppart *et al*, 2010; Hameed *et al*, 2011; Forsell *et al*, 2012), and that *Blastocystis* ST1 was the most common subtype (20%) in asymptomatic patients. The opposite phenomenon was reported by Eroglu *et al* (2009) in Turkey. In addition, Hussein *et al* (2008) found that in patients in Egypt

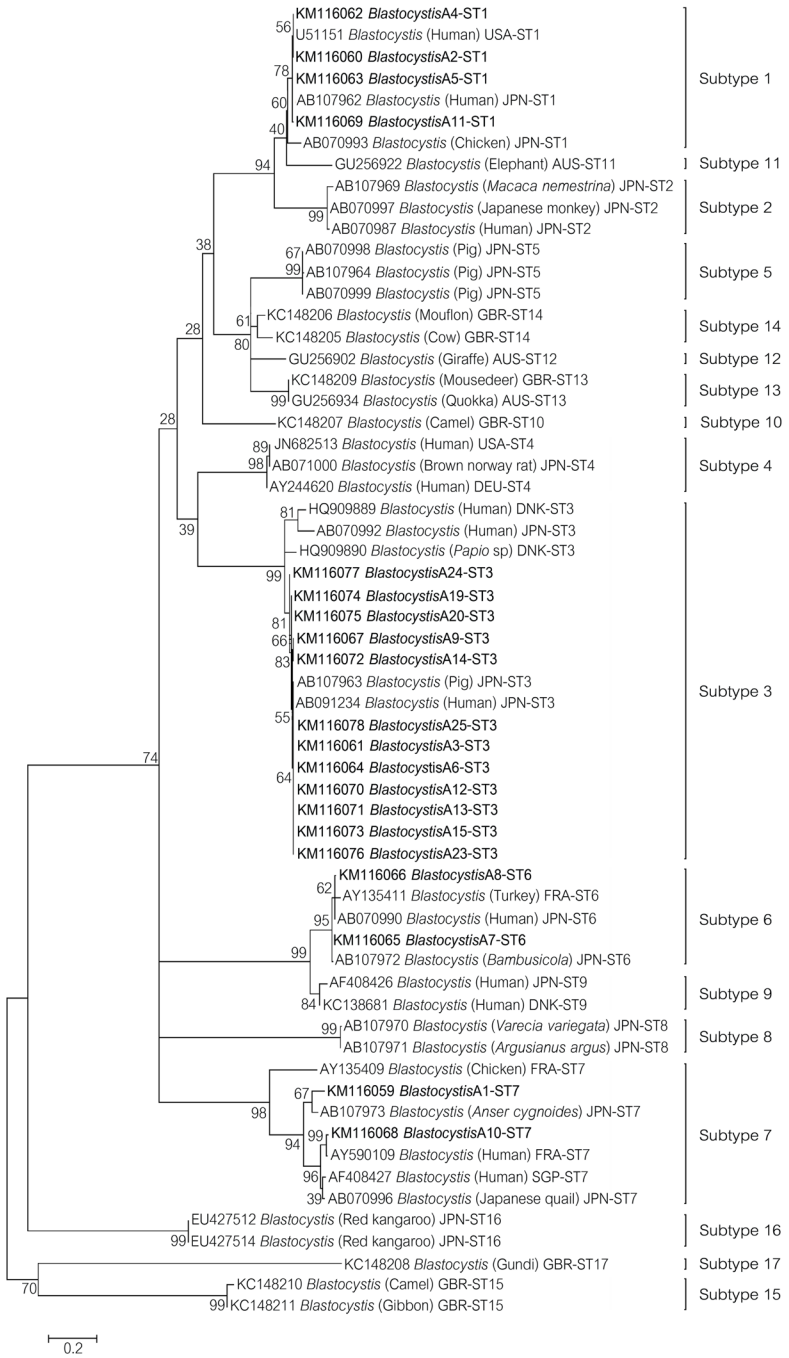

Fig 2–Maximum likelihood phylogenetic tree of *Blastocystis* subtypes based on partial 18S rDNA sequences. Sequences of *Blastocystis* subtypes 1-17 obtained from GenBank are indicated with accession number and country code (ISO 3166-1 alpha-3 codes). *Blastocystis* sequences of this study are presented in bold, and their sequences have been deposited in GenBank (KM116059-KM116078).

ST1 is clinically and statistically highly relevant to the pathogenicity of *Blastocystis* sp. The detection of *Blastocystis* STs 6 and 7 in symptomatic and asymptomatic GI patients in our study was surprising as these two subtypes have been classified as avian subtypes (Stensvold *et al*, 2009), but can be zoonotic (Yoshikawa *et al*, 2004).

It remains unclear which particular STs are responsible for the observed pathogenic effects of *Blastocystis* infection in humans. It may be difficult to understand the relationship between pathogenicity and subtype of human *Blastocystis* due to its pathogenic potential is still controversial (Yoshikawa *et al*, 2004) The present study and previous studies in Thailand have been hospital-based (Jantermtor *et al*, 2013) or in other kinds of institutions, such as at the Home for Girls, Bangkok, Thailand (Thathaisong *et al*, 2013), and future surveys of the presence and ST of *Blastocystis* in the general population should provide a clearer picture.

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