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,
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 RNA 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,
Blastocystis suspension was centrifuged at 2,500g for 20 minutes. The pellet was resuspended in 1 ml of Jones’ medium and centrifuged at 12,000g for 10 minutes, and the pellet taken up in an equal volume of 95% ethyl alcohol and stored at -70ºC 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 -20ºC 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 MgCl₂), 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: 95ºC for 4 minutes; 35 cycles of 95ºC for 30 seconds, 47ºC for 30 seconds, and 72ºC for 30 seconds; with a final heating at 72ºC 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.

### Table 1

Blastocystis isolates subtypes and patients’ biodata.

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

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 using Tamura 3-parameter (T92+G) model with uniform rates among sites, but assuming a proportion (0.23) of invariant sites.

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 Erglu 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).
STI 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.

ACKNOWLEDGEMENTS

This research was funded by the Higher Education Research Promotion of National Research University Project of Thailand, Office of Higher Education Commission through the Health Cluster (SHeP-GMS); and the Faculty of Medicine, Khon Kaen University (grant no. TR57201). Wanchai Maleewong, Pewpan M Intapan and Tongjit Thanchomnang are supported by The Thailand Research Fund Senior Research Scholar Grant (grant no. RTA5580004). The authors wish to acknowledge Professor David Blair and the Khon Kaen University Publication Clinic, Research and Technology Transfer Affairs, Khon Kaen University, for their assistance in preparing the manuscript.

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