GENETIC DIVERSITY OF *BLASTOCYSTIS* ISOLATES FROM SYMPTOMATIC AND ASYMPTOMATIC ORANG ASLI IN PAHANG, MALAYSIA

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Abstract. *Blastocystis* is a genetically diverse and widespread intestinal parasite of humans and animals with controversial pathogenic outcomes. In order to enhance our understanding of the influence of subtype differences in pathogenicity, 253 stool samples were collected from Orang Asli (aboriginal) population in Pahang, Malaysia. *Blastocystis* was identified by stool culture and subtyped by sequencing the small subunit ribosomal RNA gene. Forty-five stool samples from 21 males and 24 females, aged between 2 to 56 years, were positive for *Blastocystis* by a PCR assay, with three *Blastocystis* subtypes identified, namely, ST1 (31%), ST2 (16%) and ST3 (53%). Twelve infected [ST1 (25%), ST2 (25%) and ST3 (50%)] individuals were symptomatic [diarrhea (33%) and fever (67%)]; the remaining 33 subjects were asymptomatic. The present findings indicate that among the Orang Asli population in Pahang, Malaysia *Blastocystis* infection was likely not associated with specific subtypes, even if some subtypes were predominant in the epidemiologic studies, but rather with a conjunction of host factors, such as immune status and age. Furthermore, treatment options for *Blastocystis* should be investigated because there is no consensus as to the means of eradicating *Blastocystis* infection in this population.

Keywords: *Blastocystis*, Orang Asli, subtype, symptom, Malaysia

INTRODUCTION

Blastocystis is a single cell anaerobic and enteric parasite, which inhabits the lower gastrointestinal tract of human and many animals. This emerging parasite with a worldwide distribution is often identified as the most common eukaryotic organism reported in human stool samples and its prevalence has shown a tremendous increase in recent years (Tan, 2008). Moreover, its prevalence is higher in developing countries and this has been linked to poor hygiene practices, exposure to animals and consumption of contaminated food or water as the fecal-oral route

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is considered to be the main mode of *Blastocystis* transmission (Anuar *et al*, 2013). Indeed, a higher risk of infection has been found in humans with close animal contact, highlighting the zoonotic nature of this parasite (Yoshikawa *et al*, 2009).

Blastocystis is found in individuals both with and without symptoms. However, a report has been published of patients with gastrointestinal symptoms with *Blastocystis* as the only putative pathogen detected and whose symptoms were relieved after treatment of *Blastocystis* infection (Vogelberg *et al*, 2010). Various gastrointestinal symptoms, such as diarrhea, abdominal pain, vomiting, constipation, fever and flatulence, have been linked to blastocystosis. As with other intestinal parasitic infections, diarrheal episodes can alternate with normal defecation patterns or even constipation, conditions similar to chronic gastrointestinal illnesses such as irritable bowel syndrome (IBS) (Stensvold *et al*, 2009). Moreover, symptomatic *Blastocystis* infection has been reported to occur more often in immunocompromised adult patients than in normal individuals (Salvador *et al*, 2016). In supporting this observation, blastocystosis has also been reported to be common in HIV-infected (Tan *et al*, 2009) and immunocompromised children (Idris *et al*, 2010) with gastrointestinal symptoms.

Molecular methods have shown extensive genetic variation among *Blastocystis* isolates. A consensus approach has assigned *Blastocystis* isolates from humans, mammals and birds to one of nine subtypes (STs) with sufficient genetic divergence (Stensvold *et al*, 2007). Recently, four additional subtypes have been identified but only in non-human hosts (Stensvold *et al*, 2009). This genetic diversity has supported the hypothesis that the variability in symptomatic patients with *Blastocystis* could be due to different pathogenic potential among the different subtypes (Souppart *et al*, 2010). Hence, the main objective of the present study was to determine the level of heterogeneity of *Blastocystis* sp among symptomatic and asymptomatic Orang Asli (aboriginal) population in Pahang, Malaysia and its possible relationship with parasite's pathogenic role.

MATERIALS AND METHODS

Study area and collection of stool samples

A total of 253 stool samples from Sungai Lembing (3°55'N, 103°02'E), Pahang state, Malaysia were collected over the period from February to March 2015. Participant were asked by trained field assistants a pre-test questionnaire developed to elicit information on demographic data, socioeconomic, signs and symptoms and medical treatment. After consent was obtained and questionnaire answered, each participant then was requested to provide in a wide-mouth screw capped container pre-labeled with name and code a sufficiently large amount of stool sample to enable both microscopic examination and molecular method to be performed. Inclusion criteria were provision of informed consent and stool samples, with no limit of age group.

The study protocol [reference no. 600-RMI (5/1/6/)] was reviewed and approved by the Universiti Teknologi MARA Research Ethics Committee and permission for fieldwork was obtained from the Ministry of Rural and Regional Development Malaysia (reference no. JAKOA/PP30.052 Jld8). The study was conducted according to the Declaration of Helsinki guidelines.

Questionnaire survey

A pre-validated questionnaire was posed to participants to gather demographic data, socio-economic background, behavioral risks, living conditions and environmental sanitation and health status (history of infection and gastrointestinal symptoms). For children, their parents or guardians answered on their behalf. The participants were interviewed by four research assistants who received a specific training on how to apply the questionnaire. Alteration to the participants' normal pattern of bowel movement is defined as diarrhea if there is at least three loose stools during a 24-hour period and as dysentery if there is a passage of mucous bloody stool (WHO, 1988).

In vitro* cultivation of *Blastocystis

Approximately 5 mg of stool sample were incubated for 30 minutes at 37°C in a 5-ml screw-capped tube containing 3 ml of Jones' medium supplemented with 10% heat-inactivated horse serum (Gibco, Life Technologies, Carlsbad, CA) (Jones, 1946). Presence of *Blastocystis* was observed daily for three days by placing one drop of the culture sediment onto a glass slide pre-coated with polyvinyl alcohol, followed by staining with Wheatley's trichrome and viewed under a light microscope. Cultures were reported as positive when any of the four morphologies (vacuolar, granular, cyst, and amoeboid) of *Blastocystis* sp was observed. A negative result was recorded when there was no observed parasite growth during the incubation period.

Bacteriological and viral identification

Stool samples were also examined for the presence of other intestinal protozoans, rotavirus and adenovirus (SD Bioline Rota/Adenovirus Rapid Kit, Standard Diagnostics, Yongin, South Korea). Laboratory analyses for investigating the cause(s) of acute diarrhea included analyses for *Salmonella* spp (XLD agar), *Shigella* spp (XLD agar), *Vibrio cholerae* (TCBS agar) and enteropathogenic *Escherichia coli* (Mac Conkey and blood agar). Biochemical tests were conducted using a MICRONAUT-IDS system (MERLIN Diagnostika, Bornheim, Germany).

PCR-based detection of *Blastocystis*

Genomic DNA was extracted directly from fresh stool samples using QIAamp® Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendation procedures. In brief, approximately 0.2 g of stool sample were incubated at 70°C for 5 minutes in the cell lysis and disruption agent provided by the manufacturer, followed by proteinase K digestion. DNA was collected in a 100-µl storage solution and kept at -20°C until used. A conventional PCR assay using a specific primer set was performed as described previously (Bohm-Gloning *et al*, 1997; Stensvold *et al*, 2007). The 50 µl PCR mixture contained 25 µl of TopTaq Master Mix (QIAGEN, Hilden, Germany), 17 µl of nuclease-free water, 2 µl of each primer and 4 µl of template DNA. DNA from ST1-, ST2- and ST3-positive samples were used as the positive controls and negative control contained no DNA. Thermocycling conditions were as follows: 94°C for 3 minutes; 35 cycles of 94°C for 30 seconds, 59°C for 30 seconds and 72°C for 60 seconds; with a final step at 72°C for 10 minutes. Amplicons (550-585 bp) were analyzed by 1.5% agarose gel-electrophoresis and stained with GelRed (0.1 µl/ml; Biotium, Hayward CA).

DNA sequencing and phylogenetic tree construction

Amplicons were sequenced in both directions using the same PCR primer set as

Table 1
Microscopic examination and PCR assay for *Blastocystis* detection and subtyping performed on stool samples.

	PCR[b]				
	ST1	ST2	ST3	Negative	Total
Microscopic examination[a]					
Positive	11	5	18	33	67
Negative	3	2	6	175	186
Total	14	7	24	208	253

[a]Subtype cannot be distinguished. [b]Subtype detected by PCR and phylogenetic analysis. ST1, subtype 1; ST2, subtype 2; ST3, subtype 3.

using an ABI 3730XL sequencer (Applied Biosystems, Foster City, CA). Forward and reverse sequences were edited and manually aligned, and a consensus sequence was created for each sample using BioEdit Sequence Alignment Search Tool (BLAST) for comparison with the National Centre for Biotechnology Information (NCBI) reference sequences. Subtypes were identified by determining exact match or closest similarity according to the classification of Stensvold *et al* (2007). A phylogenetic tree was constructed using the maximum likelihood method employing MEGA software version 6.06. Flagellate *Proteromonas lacertae* (GenBank accession no. U37108) was used as the outgroup. Bootstrap confidence values for branching reliability were calculated with 100 replicates. The small subunit ribosomal DNA (SSU rDNA) sequences obtained in this study were deposited in GenBank under accession nos. KX108706, KX108712, KX108713, KX108714, KX108718, KX108723, and KX108731.

RESULTS

Of the 253 stool samples, 67 were microscopy-positive for *Blastocystis* of which 33 were negative by the PCR assay, whereas among the 186 microscopy-negative samples 11 were PCR-positive (Table 1). Among the 33 PCR-negative samples in the former group, 14 were positive for vacuolar form, 12 granular, 3 both vacuolar and granular forms, and 1 each was positive for cyst, ameboid, both granular and amoeboid, and both granular and cyst. Of the 45 PCR-positive samples, 21 were males and 24 females, aged between 2 to 56 years.

Sequences of the 45 amplicons showed 97-100% similarity to *Blastocystis* sequence in GenBank (accession no. AB070988). Twelve and 33 *Blastocystis*-positive samples were from symptomatic and asymptomatic individuals, respectively, and four individuals had *Blastocystis* in association with pathogenic protozoans (Table 2).

Six ST3 were detected among symptomatic individuals, followed by ST1 (3) and ST2 (3) (Fig 1). In addition, 18 ST3, 11 ST1 and 4 ST2 were found in stool samples from asymptomatic individuals. All symptomatic Orang Asli subjects positive for *Blastocystis* infection were negative for the presence of both viral (adenovirus and rotavirus) and bacterial (*Salmonella*

Table 2
Clinical details of twelve symptomatic Orang Asli individuals positive for *Blastocystis* by microscopy and PCR.

Age (year)	Sex	Microscopy	Sub-type	Symptom	Virus/bacteria	Other intestinal protozoa
32	M	Positive	ST1	Diarrhea	Negative	None
11	F	Negative	ST1	Fever, dyspepsia	Negative	None
20	F	Positive	ST1	Diarrhea	Negative	*Entamoeba histolytica*
56	M	Positive	ST2	Fever, dyspepsia	Negative	*Endolimax nana*
48	M	Negative	ST2	Fever, abdominal pain	Negative	*Entamoeba hartmanni*
14	F	Positive	ST2	Fever, nausea/ vomiting	Negative	*Giardia intestinalis*
2	M	Positive	ST3	Fever	Negative	*Endolimax nana, Giardia intestinalis*
20	F	Positive	ST3	Fever, abdominal pain, flatulence	Negative	*Entamoeba hartmanni*
15	F	Positive	ST3	Fever, flatulence	Negative	None
6	F	Negative	ST3	Fever, abdominal pain	Negative	None
22	M	Positive	ST3	Diarrhea	Negative	*Endolimax nana, Giardia intestinalis*
28	F	Positive	ST3	Diarrhea	Negative	*Entamoeba coli*

Subtypes were determined by PCR.

sp, *Shigella* sp, *Vibrio cholerae* and enteropathogenic *Escherichia coli*) pathogens.

DISCUSSION

It is still a matter of debate whether blastocystosis is subtype related. Clark (1997) was the first to suggest *Blastocystis* subtype correlates with the parasite's pathogenic potential. In this study, the identity of *Blastocystis* subtypes present in the Orang Asli population was investigated by PCR and sequencing, as well as its association with clinical manifestations of infection. It was anticipated that the present findings would provide further knowledge on the pathogenicity of the different subtypes, as well as a link to human gastrointestinal disease. This study reveals tST3 was the most predominant *Blastocystis* subtype isolated from stool of symptomatic and asymptomatic Orang Asli and is suggested to be of human origin. Epidemiological studies from other locations around the world indicated ST3 is commonly but not exclusively the most predominant subtype isolated from symptomatic and asymptomatic individuals (Ozyurt *et al*, 2008; Souppart *et al*, 2009). Likewise, Hameed *et al* (2011) reported presence of ST3 from the symptomatic *Blastocystis* urticarial patients in Cairo, Egypt. On the other hand, Yoshikawa *et al* (2004) did not find any association between the more common subtype ST3 and clinical manifestations, which was confirmed by Kaneda *et al* (2001). Approximately 25% of *Blastocystis* ST3-infected Orang Asli individuals presented some sort of

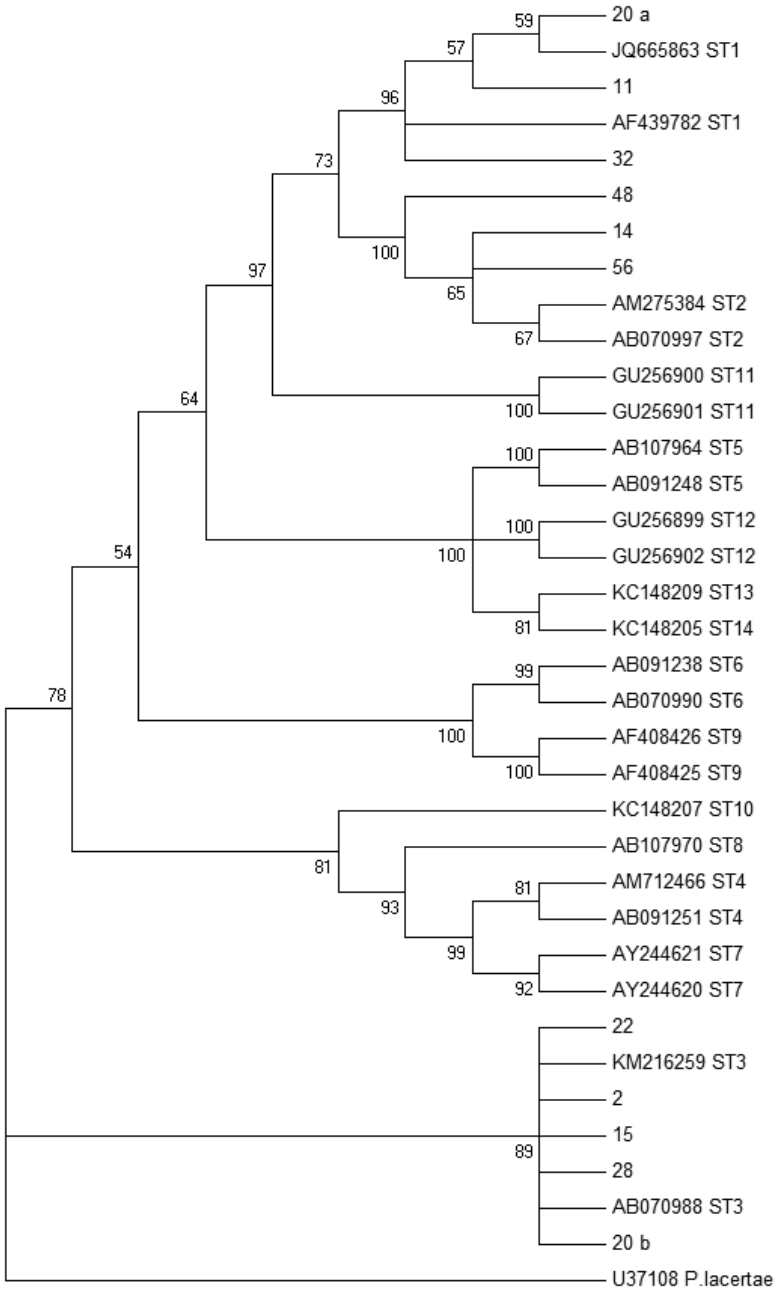

Fig 1-A phylogenetic tree of *Blastocystis* small subunit rDNA (partial) sequences from stool of symptomatic Orang Asli individuals and reference sequences from GenBank, shown with accession numbers. The tree is inferred using the maximum likelihood method with bootstrap values indicated at the internal nodes (100 replicates). The position of the subtypes in the tree is indicated on the right of the tree. *Proteromonas lacertae* (accession number U37108) was used as the outgroup.

gastrointestinal symptoms, with the strongest association being with diarrhea and fever. Although clinical features of illnesses, such as diarrhea, abdominal pain, nausea, fatigue, constipation, flatulence, and vomiting that have been attributed to *Blastocystis* are nonspecific, several studies have reported abdominal pain and diarrhea are two of the major symptoms in *Blastocystis*-positive patients (Tan, 2008).

Surprisingly, three ST2 isolates in our survey were associated with symptoms. Although one of these three Orang Asli subjects had another pathogenic intestinal protozoon present (*Giardia intestinalis*), the other two individuals had no other infectious agents. There is conflicting evidence regarding the pathogenicity of ST2, with two studies showing high infection intensity of ST2 (Ozyurt *et al*, 2008; Vogelberg *et al*, 2010), while another study demonstrated no link between ST2 and pathogenicity (Yan *et al*, 2007). To the best of our knowledge, this is the first time that ST2 strains have been isolated from

symptomatic Orang Asli population. The present study also confirmed ST1 strains could be associated with pathogenicity as suggested in previous studies carried out in other countries (Dominguez-Marquez *et al*, 2009; Souppart *et al*, 2009). Hussein *et al* (2008) confirmed this hypothesis by concluding that ST1 is the most virulent, while ST3 consists of both pathogenic and non-pathogenic strains. ST4 was not found among symptomatic and asymptomatic individuals, consistent with previous study of Souppart *et al* (2010). On the other hand, ST4 was identified as the most common subtype (94.1%) in a large symptomatic group of patients in Spain (Dominguez-Marquez *et al*, 2009). ST4 was particularly absent in subtropical countries, suggesting that infection with ST4 is dependent on the geographical area (Alfellani *et al*, 2013).

Almost all data available from epidemiological surveys of symptomatic individuals including this study confirmed that several *Blastocystis* subtypes could be involved in human infections associated with gastrointestinal symptoms, and that the implicated subtypes and their relative frequency might broadly differ among countries as well as within the same country. For instance, in Turkey, remarkable differences were observed among communities as four subtypes (ST1-4) were detected (Ozyurt *et al*, 2008), while ST1 is the only subtype detected in 20 symptomatic patients (Eroglu *et al*, 2009). In Egypt, a total of three subtypes (ST1-3) were identified in symptomatic patients (Souppart *et al*, 2009). In one local study, at least 4 different subtypes (ST1-4) were found in cancer and HIV-infected patients with gastrointestinal symptoms (Tan *et al*, 2009). In previous studies, the existence of pathogenic and non-pathogenic variants in different subtypes including ST3 was also suggested (Ozyurt *et al*, 2008; Souppart *et al*, 2009).

In 33 samples positive for *Blastocystis* by microscopy, PCR failed to amplify DNA from any member of the *Blastocystis* subtypes assayed with the primer pairs employed and there was no inhibition of the PCR in control experiments. These results might be explained by the presence of DNA polymerase inhibitors in stool, degradation of DNA due to long storage time or the presence of a too low number of parasites in the sample. Another possible explanation is the possibility of culture subtype preference thereby supporting growth of certain subtypes that are not recognized by the set of PCR primers used.

In summary, it is clear from the present study that only an association and not necessarily causality, indicating a lack of correlation between specific subtype and pathogenic potential of *Blastocystis* sp. Analysis of *Blastocystis* DNA sequences from different groups of population is mandatory if there is to be any attempt to investigate the potential pathogenicity of this parasite. Furthermore, the pathogenic role(s) of *Blastocystis* sp should not be dismissed lightly especially in cases where other pathogenic protozoa, helminthes, bacteria, and viruses are not detected. Further studies should be continued to provide information on *Blastocystis* subtypes and sequence data, preferably as part of case-control studies.

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