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OXFORD

Diagnosis and Identification of *Blastocystis* Subtypes in Primary School Children in Jakarta

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ABSTRACT

Blastocystis hominis is an enteric protozoan with many subtypes. It is frequently found in children and may cause chronic diarrhea. This study revealed *Blastocystis* subtypes among primary school children and comparison of molecular technique and culture method in *Blastocystis* diagnosis. A total of 141 stools were collected, examined microscopically, selected into the *Blastocystis* and negative parasite groups, for diagnostic comparison between culture and 18S rRNA polymerase chain reaction (PCR) methods. Positive PCR amplicons were subsequently sequenced for subtyping. The PCR results revealed 89%, 78%, 80% and 88% sensitivity, specificity and positive and negative predictive values, respectively, in comparison with the culture method (McNemar, $p > 0.05$). Sixteen PCR samples were successfully sequenced and resulted in three *Blastocystis* subtypes 1, 3 and 4. In conclusion, PCR was sensitive enough and can be used to exclude *Blastocystis* infection up to 88% of the cases. Subtypes 3 and 1 were the main subtypes found in apparently healthy school children in Jakarta.

KEYWORDS: intestinal parasite, children, diagnostic, *Blastocystis* subtype

INTRODUCTION

Blastocystis hominis is an intestinal protozoan, infecting humans as well as animals such as mammals, amphibians, reptiles and arthropods. It is distributed widely in the world and transmitted via fecal-oral route through ingestion of cysts [1]. *Blastocystis* prevalence in children could reach 20–30% or even >50% as reported by Kurniawan and Pegelow [2, 3] in a survey among primary school children in North Jakarta and Sukaraja, respectively. The lowest prevalence of 0.5% was reported in Japan [4].

Blastocystis is often regarded as commensal or nonpathogenic and frequently found in epidemiological surveys. Some regarded *Blastocystis* as an opportunistic pathogen among the immune compromised while some epidemiological data support pathogenic characteristic of *Blastocystis* [5, 6]. The clinical symptoms of *B. hominis* infection may manifest as bloating, anorexia, abdominal pain, acute and chronic diarrhea or even 'irritable bowel syndrome'. Chronic diarrhea in children will consequently disturb their growth and development particularly among those <5 years old [7].

Diagnosis of *Blastocystis* can be carried out microscopically; however, the result is subjective and depends on the technician's experience and skill.

The size and morphology of *Blastocystis* varies widely during developmental cycle; vacuolar form, cyst, amoeboid, granular, multivacuolar and avacuolar, which may mimic other intestinal protozoa or contaminants and subsequently cause misdiagnosis. A number of studies have reported other methods such as culture using ATCC Medium 1671, Locke Medium or Jones medium and polymerase chain reaction (PCR) to identify *Blastocystis* infection [8], and culture method was regarded as the gold standard for *Blastocystis* diagnosis [9].

As *Blastocystis* has a wide range of hosts, the genetic analysis of SSU rRNA from human, mammals, primates and birds reveals at least 17 subtypes of *Blastocystis* with subtype 3 (ST3) as the most common subtype found in human. Some subtypes (ST1-9) commonly seen in human can also be found in animals, suggesting potential zoonotic transmission [10].

This study aimed to compare the DNA detection by PCR and culture by Jones medium, determine the sensitivity and specificity of PCR to be used as diagnostic tool and to obtain the genetic profile of *Blastocystis* subtypes among the school children. Ethical clearance was obtained from the ethics committee, Faculty of Medicine, Universitas Indonesia and informed consents from the participants and/or the teachers.

MATERIALS AND METHODS

Study population

A cross-sectional study was carried out among primary school children in Kampung Melayu, East Jakarta. The study area was densely populated and vulnerable to flood from Ciliwung river every year. As many as 141 students were recruited from two primary schools. Fecal samples were collected, transported and examined at Parasitology laboratory, Faculty of Medicine, Universitas Indonesia. The samples were divided into three parts: first part for direct smear examination, second part for culture and the third part were kept in −20°C until it was used for molecular analysis.

Parasitology examination and culture

Stools were examined directly with 0.2% Lugol solution under microscope to detect any intestinal parasites. To perform the diagnostic test comparison between culture method and molecular detection for *B. hominis* diagnosis, the stools were further screened for single infection of *B. hominis* (*Blastocystis* group) and no parasites (negative group): 18 samples in each group following sample size estimation formula for diagnostic test. Stools from the two groups were cultured in Jones medium supplemented with 10% horse serum [11], incubated at 37°C and kept for 72 h before reporting as positive or negative *Blastocystis*. Examination of culture was performed after 24 h incubation.

DNA isolation and amplification

Stool samples from the two groups (the *Blastocystis* and negative groups) were DNA extracted using QIAamp DNA Mini Kit following the procedure from the manufacturer (Qiagen, GmbH, cat no. 51306). The final isolated DNA was resuspended in 90 µl AE buffer and kept in −20°C.

Extracted DNA was PCR amplified in 20 µl reaction using TopTaq Polymerase Master Mix Kit (Qiagen, GmbH, cat no. 200403), which contains Taq DNA Polymerase, PCR Buffer (with 1.5 mM $MgCl_2$), 200 µM each dNTP, 10% BSA (Bovine Serum Albumin) to minimize the inhibitors effect and 2 µl DNA template with the following primers as previously described in [12].

Forward: RD5 5′-ATC-TGG-TTG-ATC-CTG-CCA-GT-3′
Reverse:. BhRDr 5′-GAG-CTT-TTT-AAC-TGC-AAC-AAC-G-3′

DNA amplification was performed by direct PCR targeting 18S rRNA gene. All PCR reactions were carried out on MJ Research PTC 200 thermocycler as follows: one cycle at 95 °C for 6 min followed by 30 cycles at 93 °C for 2 min, 65 °C for 2 min, 72°C for 2 min. The last cycle was extended for 10 min at 72 °C. The 600 bp PCR product was visualized on 2% agarose gel electrophoresis.

DNA sequencing and subtype analysis

To characterize genetic diversity of *Blastocystis*, all PCR products from *Blastocystis* positive samples were directly sequenced and chromatograms were validated

using Chromas lite software (Technelysium Pty Ltd, Australia). Blasted sequences were multiple aligned with *Blastocystis* database retrieved from the GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi), using Mega 6 software (en.bio-soft.net/tree/MEGA.htm).

Statistical analyses

The data were statistically analyzed using Cat Maker® software [13], with p value ≤ 0.05 regarded as significant. Accuracy was calculated as specificity, sensitivity and positive and negative predictive values, with 95% confidence intervals (CI).

RESULTS

Prevalence of *B. hominis*

As many as 141 samples were collected, and direct fecal smear examination revealed that 74 (52.5%) children were positive for intestinal parasites. Single infection of *B. hominis* was observed in 47 children (33.3%), mix infection of *B. hominis* in 11 children (7.8%) and 16 children (11.3%) infected with other intestinal parasites (Table 1). The mix infection of *B. hominis* can be with *Giardia duodenalis*, *Entamoeba coli*, *Entamoeba histolytica* or *Trichuris trichiura*. The overall prevalence of *Blastocystis* in the study population was 41.1% where 80% infection was present among the 1st–3rd year students. This result suggested high prevalence of parasitic infection among the primary school children in Kampung Melayu area, East Jakarta which was dominated by the protozoa *B. hominis* and *G. duodenalis* (Table 1).

Comparison of PCR and culture methods in *Blastocystis* diagnosis

The culture showed 100% agreement with direct examination result in both groups, the *Blastocystis* and negative groups. The PCR result showed 89% sensitivity (95% CI: 74–100%) and 78% specificity (95% CI: 59–97%), 80% positive predictive value and 88% negative predictive value in comparison with the culture method (Table 2). Statistical analysis showed no significant difference between those two methods in diagnosing *Blastocystis* infection (McNemar, $p > 0.05$).

Blastocystis subtype analysis

There were 16 of 18 samples positive for *Blastocystis*, which were successfully amplified targeting the 18S rRNA gene and sequenced. Sequences were deposited in Genbank with accession numbers JN 682513.1, KP 233714.1, KP 233717.1, KP 233722.1, KP 408437.1, KP 408442.1, KP 408449.1, KP 408450.1, KT 438687.1 and LT 222066.1. The result demonstrated five samples of subtype 1 (ST1), 10 samples of subtype 3 (ST3) and one of subtype 4 (ST4); ST3 was the major subtype (Figure 1). Only subtype 4 (ST4) found in this study perfectly matched the genbank sequence database. A phylogenetic tree of the *Blastocystis* isolates is displayed in Figure 1, using the neighbor-joining method with the bootstrap proportions (%) attached to the internal branches from 500 replicates.

DISCUSSION

This study showed a high prevalence of intestinal parasitic infection dominated by *Blastocystis* among the primary school children living in a densely populated area by Ciliwung river in East Jakarta, which always flooded every year during rainy season. This prevalence is similar to other studies performed among primary school children in Sumba, rural area in East Indonesia, North Jakarta and Sukaraja with the exception that higher soil-transmitted helminthiasis were found in the other studies [2, 3, 14]. Flood, which happened every year during the rainy season in that area, may become one factor that contributes to the high prevalence of *Blastocystis* apart from personal hygiene factor of the children, as noted in this study that 80% *Blastocystis* infection happened among younger students, aged 6–9 [15]. This urges the importance of continous health education and practice among the primary school students to prevent parasitic infections.

Diagnosis of *Blastocystis* in this study used direct microscopic examination, culture and PCR on 18S rRNA gene. Culture and direct PCR methods from fresh stool samples were tested because direct smear examination has weakness in *Blastocystis* diagnosis because of diversity in morphology and size of *Blastocystis*, which may mimic other parasites or organisms or contaminant in the stool [8, 16]. This study did not show superiority of PCR against the culture

Table 1. Intestinal parasites profile among primary school children in East Jakarta

Location	Single infection					Mixed infection				Negative	Total
	B. hominis	*G. duodenalis*	*E. coli*	*Ascaris lumbricoides*	*T. trichiura*	Bh+Gd	Bh+Tt	Bh+Ec	Bh+Eh		
School A	23	5	2	1	1	1	2	0	0	31	66
School B	24	6	1	0	0	3	0	4	1	36	75
Total	47	11	3	1	1	4	2	4	1	67	141
(%)	(33.3)	(7.8)	(2.1)	(0.7)	(0.7)	(2.8)	(1.4)	(2.8)	(0.7)	(47.5)	(100)

Notes: Bh + Gd: *B. hominis* + *G. duodenalis*.
Bh + Tt: *B. hominis* + *T. trichiura*.
Bh + Ec: *B. hominis* + *E. coli*.
Bh + Eh: *B. hominis* + *E. histolytica*.

Table 2. Comparison between culture and PCR in Blastocystis diagnosis

		Culture		Total	*p* value
		Positive	Negative		
PCR	Positive	16 (44.4%)	4 (11.1%)	20 (55.6%)	
	Negative	2 (5.6%)	14 (38.9%)	16 (44.4%)	0,687
Total		18 (50%)	18 (50%)	36 (100%)	

method, which is in contrast to the statement from Stensvold *et al.* [16] that the sensitivity of culture is in 52–79% compared with DNA-based methods. Four samples that were negative by culture turned out to be positive by PCR, and two samples that were positive by culture turned out to be negative by PCR; however, statistical analysis did not show significant difference between those two methods. Several reasons may explain this finding. First, the DNA that was isolated from fresh stool may have less quality and quantity than those isolated from culture [5, 16].

Stensvold *et al.* [16] reported that PCR sensitivity will increase three times when using *Blastocystis* DNA isolated from culture than from the fresh stool. Secondly, the presence of inhibitors such as bile salts, polysaccharide complex in stool specimens will subsequently lower the DNA recovery or the amount of *Blastocystis* DNA was below detection level [5, 17].

However, with 88% negative predictive value, PCR can be used to exclude suspected *Blastocystis* infection up to 90%.

The result of 16 of 18 *Blastocystis* positive stool samples, which were successfully amplified, sequenced and subtyped, yielded dominance of ST3 (67.95%), followed by ST1 (26.4%), ST2 (3.8%) and the least was ST4 (1.9%) in only one sample. Dominance of ST3 *Blastocystis* in human infection is in agreement with other previous reports in Europe, Africa, Asia and Australia, except in America, which was dominated by ST1 [1]. There have been several reports on *Blastocystis* subtypes from Indonesian population, which showed only subtypes 1, 2 and 3 in which ST3 predominated in the studies among migrant workers in Taiwan and Qatar [18, 19], while ST1 predominated among children in Sumba, East Nusa Tenggara [13]. ST4 is the rarest subtype among Asian people except Nepalese; however, it is quite prevalent and predominant among the Spanish, British and Australian [1, 10, 20] and was reported to be associated with acute diarrhea among Danish patients [21]. ST4 commonly infects rodents, marsupials, ratites and primate [22]. A study in Sumba, Indonesia by Yoshikawa *et al.* [14] showed that ST4 was observed only in wild rodents and none among the residents who live together with domestic and wild animals, and postulated the presence of host specificity and difficulty for zoonotic transmission, which is in contrast to other studies that

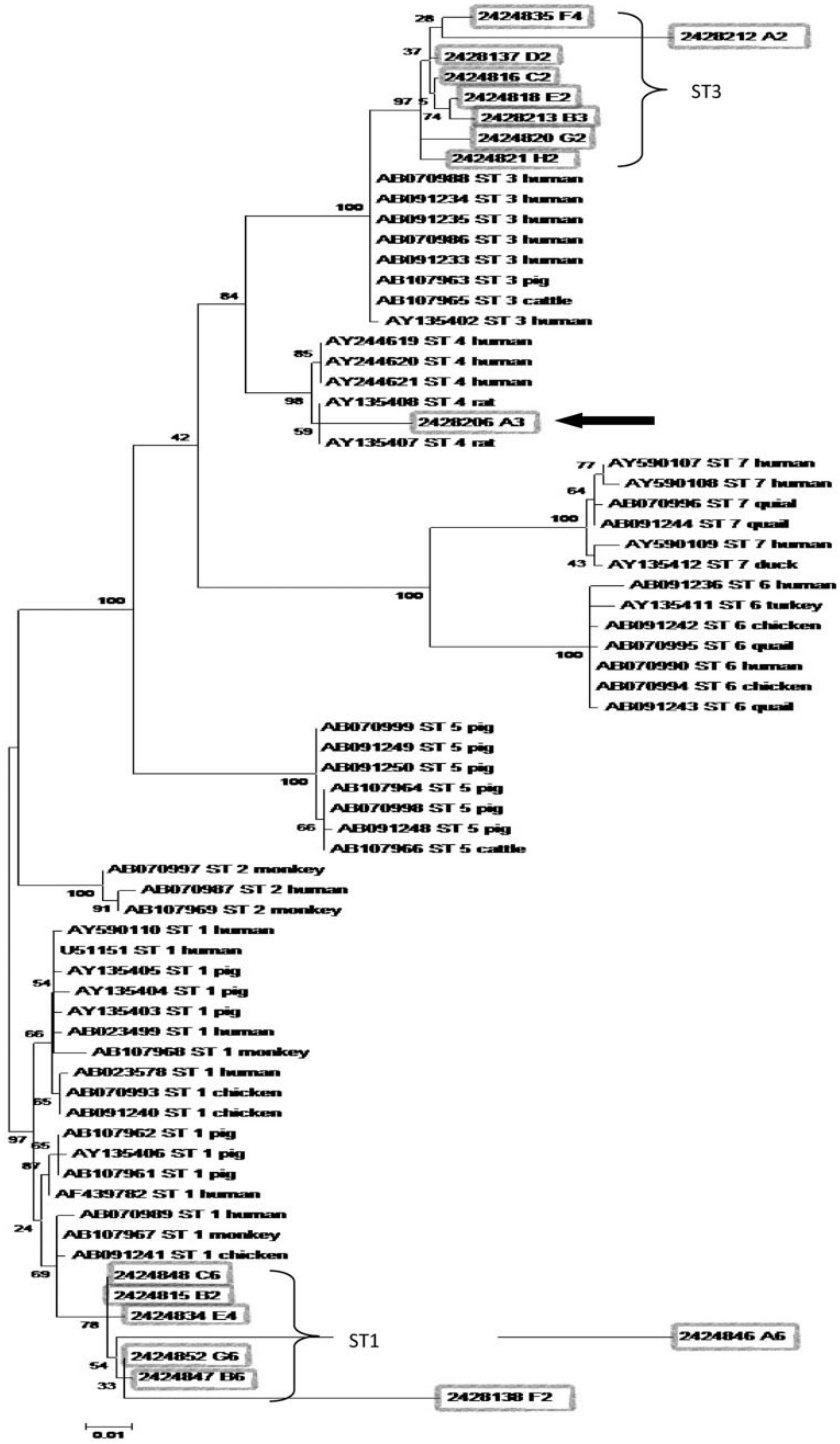

Fig. 1. Phylogenetic tree analysis of *Blastocystis* samples sequences on 18S rRNA.

suggested the role of rodents as reservoir host for zoonotic *Blastocystis* infection in human [5, 23, 24].

The finding of ST4 in our study showed that ST4 is an emerging *Blastocystis* subtype infecting human population in Asia, a minor subtype, which has been also observed among the school children in Malaysia and Turkey [25, 26]. The possibility of zoonotic transmission of ST4 from rat to human (the student) in our study cannot be avoided owing to the densely populated area where the student lives and rat is a common animal found in the surrounding area. Unlike ST3 and ST1, which can be found in symptomatic and asymptomatic infection, the presence of ST4 should raise concern and association to the possibility of pathology, which needs further study to clarify any association between particular subtype and clinical manifestation.

In conclusion, high prevalence of *Blastocystis* infection is seen among the primary school children in East Jakarta, with ST3 as the most common subtype. PCR can be used to exclude up to 88% cases of suspected *Blastocystis* infection.

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