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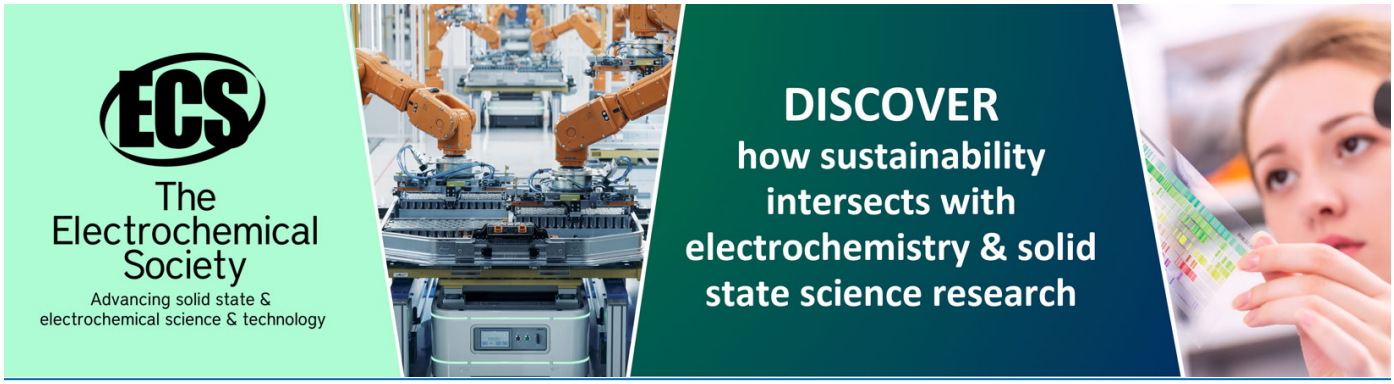

Association of *Blastocystis* subtypes with diarrhea in children

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Abstract. *Blastocystis hominis* is an intestinal zoonotic protozoa that epidemiological surveys have shown, is highly prevalent among children and may cause chronic diarrhea. This study aimed to identify *Blastocystis* subtypes among children and associate those subtypes to pathology. The study's population was children aged 6–12 years old divided into asymptomatic and symptomatic (diarrhea) groups. The asymptomatic samples were obtained from primary school students in the Bukit Duri area of South Jakarta, while the symptomatic samples were obtained from patients who visited nearby primary health centers (Puskesmas). Symptomatic stool samples were examined inParasitology Laboratory FKUI. Microscopic examination of the stool samples was performed to screen for single *Blastocystic* infection, followed by culture, PCR of 18S rRNA, and sequencing. In the study, 53.2% of children (n = 156) harbored intestinal parasites, *Blastocysts sp.* A single infection of *Blastocystis sp.* was present in 69 (44.23%) samples, comprised of 36 symptomatic and 33 asymptomatic participants. The *Blastocystis* subtypes (STs) identified in this study were STs 1–4; ST3 was the most dominant and was observed with statistically significant higher frequency in the symptomatic group. ST4 was only found in one sample in the symptomatic group. While ST1 and ST2 were found more frequently in the asymptomatic group, no statistical association was observed. ST3 is more likely to be associated with clinical symptoms than ST1 and ST2.

1. Introduction

Blastocystis hominis is a zoonotic, micro-eukaryote parasite commonly found in the intestinal tract of humans and animals, such as mammals, amphibians, reptiles, and insects. Infection caused by this intestinal parasite has become a significant public health issue in developing countries. Despite the rate of mortality caused by *Blastocystis hominis*, infection is relatively low. The infection results in symptoms such as chronic diarrhea, malnutrition, nutrition-related physiological changes, and mental developmental issues among children [1]. Epidemiological surveys have shown that *Blastocystis hominis* is distributed worldwide in tropical and sub-tropical regions in children as well as adults, with higher prevalence in developing countries (30–60%) than developed countries (1.5–10%) [1-3]. This contrast in pervasiveness can be linked to standards of hygiene, waste disposal, exposure to animals, and consumption of food or water contaminated by the parasite [1].

The pathogenic potential of *Blastocystis* is controversial because the infection can be asymptomatic or have clinical manifestations. Symptoms associated with *Blastocystis* infection include bloating, nausea, abdominal pain, anorexia, acute to chronic diarrhea, or symptoms commonly associated with

irritable bowel syndrome (IBS); it is also an opportunistic pathogen in immunocompromised patients. While some epidemiological data strongly suggest that *Blastocystis hominis* is a pathogen [4], other studies suggest *Blastocystis* is a commensal parasite in human intestines [5]. *Blastocystis* infection is caused by poor hygiene, especially among elementary schoolchildren. Chronic diarrhea and repeated infections can affect children's growth and development [6].

Conflicting views about the pathogenicity of *Blastocystis hominis* may be related to the number of infecting parasites present, the duration of the infection (acute or chronic), the host's genetic factors, or the different subtypes or species of *Blastocystis hominis* [5]. Based on gene analysis of small subunit ribosomal RNA (SSU rRNA), 17 subtypes (STs) have been identified in humans, primates, mammals, and birds; ST1–ST9 are found in humans, with ST3 being the most common. However, human *Blastocystis* subtypes have also been identified in animals, suggesting that animals may act as reservoirs for *Blastocystis* and may be linked to zoonotic transmission [7]. This study aimed to identify the subtype distribution and dominant subtype(s) of *Blastocystis* among symptomatic and asymptomatic schoolchildren and associate those subtypes to pathology.

2. Materials and Methods

2.1. Study population

The population studied was primary schoolchildren aged 6–12 years old in the Bukit Duri district of South Jakarta. The population was divided into asymptomatic and symptomatic (diarrhea) groups; 32 samples for each group were obtained following the sample size estimation. The inclusion criteria for asymptomatic samples were as follows: children aged 6–12 years old with no gastrointestinal symptoms (nausea, diarrhea, abdominal pain) in the preceding four weeks and normal stool consistency with a single infection of *Blastocystis* upon parasitologic examination. The symptomatic samples were obtained from pediatric patients diagnosed with diarrhea and gastroenteritis at two primary health centers (Puskesmas) in the Bukit Duri district. Any children who were primarily included in the asymptomatic group who subsequently visited a Puskesmas due to diarrhea were excluded from the study. After being informed about the study, informed consent was obtained from the patients or their parents or school teachers. Ethical clearance was obtained from the Ethics Committee of the Faculty of Medicine at the Universitas Indonesia (document no: 867/UN2.F1/ ETIK/2016).

2.2. Parasitology examination

Fresh stools collected from the field were taken to the Laboratorium Parasitologi FKUI and screened microscopically for consistency and the presence of blood and mucus, then directly tested microscopically using a 1% Lugol solution. Samples determined to be *Blastocystis* positive were subsequently cultivated in Jones medium to increase their amount. Fresh samples of 200 µl were cultivated in 1800 µl of Jones medium supplemented with 200 µl of horse serum and incubated at 37 °C for 48 hours. The cultivated samples were pelleted and stored at −30 °C for DNA extraction.

2.3. PCR and sequencing

DNA extraction was performed using a QiaAMP DNA Stool Mini Kit [QIAGEN, USA, cat. no. 51306] according to the manufacturer's protocol. The extracted DNAs were resuspended in 90 µl of AE buffer, then stored at −20 °C for further analysis. Target gene amplification using PCR were obtained according to the procedures explained in a previous study by Scicluna *et al*. [8] PCR reaction using forward primer RD5: 5' GAG CTT TTT AAC TGC AAC AAC G 3' and reverse primer BhRDr: 3' ATC GGT TTG ATC CTG CCA GT 5' were employed in a 20 µl PCR reaction. The amplification product was a 600 bp fragment of the 1800 bp of an SSU rRNA gene. The master mix per test consisted of 1x PCR buffer, 2.5 mM MgCl2, 200 µM each of dNTP (dATP, dGTP, dTTP, dCTP), 250 nM of each primer, 1.25 U of Taq polymerase, 0.5 µl of BSA, and 2 µl of extracted DNA. Amplification was performed using an MJ Research PTC 200 thermocycler as follows: one cycle at 95 °C for 5 min, followed by 35 cycles at 93 °C for 2 min, at 65 °C for 2 min, and elongation at 72 °C for 2 min. The final extension step was at 72 °C for 10 min. The positive control employed was *Blastocystis hominis* DNA. The PCR amplicon was

electrophoresed on 2% agarose gel, run at 100 V for 1 hour in TBE buffer and stained with AtlasSight DNA Stain (BioatlasTM) with a 100 bp DNA ladder. The result was visualized using a UV transilluminator.

2.4. DNA sequencing and subtype analysis.
Subtype determination of the *Blastocystis* was performed by direct sequencing, and chromatograms were validated using Chromas Lite software 9 (Technelysium Pty Ltd, Australia). Sequence analysis was performed using Mega 6 software; sequences were blasted and multiples aligned with the *Blastocystis* database were retrieved from the GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5. Statistical analysis
Statistical analysis was performed using SPSS version 20. Pearson's chi-squared test (X2) and Fisher's exact test were used to test the correlation between *Blastocystis* infection and related factors; $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1 Results

3.1.1. Blastocystis infection profile in primary schoolchildren
A total of 156 stools were collected from primary schoolchildren, 73 samples from male students and the remainder from female students. The samples were examined microscopically, and the results showed that 53.2% of children had intestinal parasites, predominantely*Blastocystis sp*. A single infection of *Blastocystis sp.* was present in 69 (44.23%) samples, comprised of 36 symptomatic and 33 asymptomatic subjects. Other intestinal parasites found were *Giardia duodenalis, Trichuris trichiura*, and *Entamoeba coli* (Table 1). The infection rate was higher among children 6–9 years old (65.2%) compared to those aged 10–13 years old (34.8%); the difference was not statistically significant ($p = 0.792$, $p > 0.05$).

Table 1. Intestinal parasite infection profile in primary school children

Characteristic	***Blastocystis* Infection**	Mix infections	Other Infections	Uninfected	Total
Symptomatic	36 (63.1%)	0 (0%)	3 (5.3%)	18 (31.6%)	57 (100%)
Asymptomatic	33 (33.3%)	1 (1.0%)	8 (8.1%)	57 (57.6%)	99 (100%)
Total	69 (44.2%)	1 (0.6%)	11 (7.1%)	75 (48.1%)	156 (100%)

3.1.2. Subtype of Blastocystis in symptomatic and asymptomatic groups
PCR was performed on all *Blastocystis* positive samples, and the whole samples showed positive results, suggesting that the 18S rRNA target gene was successfully amplified. Four subtypes (ST1–ST4) were successfully determined; ST3 was the most frequent subtype, followed by ST1 and ST2 (Table 2). The ST3 *Blastocystis* subtype was also observed more frequent among those withclinical symptoms than ST1 and ST2. Statistical analysis showed no significant difference between ST1 and ST2 in causing clinical symptoms (chi-square test, $p = 0.3$); however, there was a significant statistical difference between ST2 and ST3 (Fisher's exact test, $p = 0.036$) and between ST1 and ST3 (Chi-square test, $p = 0.00$). These results suggest that ST3 has more pathogenic potential than ST1 and ST2.

Table 2. *Blastocystis* subtype in symptomatic and asymptomatic children

Group	ST1	ST2	ST3	ST4	ST5-9	Total
Symptomatic	8 (22.2%)	1 (2.8%)	26 (72.2%)	1 (2.8%)	0	36 (100%)
Asymptomatic	11 (33.3%)	4 (12.1%)	18 (54.6%)	0	0	33 (100%)
Total	19 (27.5%)	5 (7.2%)	44 (63.8 %)	1 (1.5%)	0	69 (100%)

3.2 Discussion

Microscopic diagnosis of the samples indicated that 83 of 156 (53.20%) samples were infected by gastrointestinal parasites such as *Blastocystis sp., Giardia intestinalis, Trichuris trichiura*, and *Entamoeba coli*. This infection rate was slightly lower than the 64.6% prevalence rate shown in a a previous study amongelementary school students in Bekasi, a satellite city of Jakarta [9]. The infection rate of *Blastocystis* did not differ between this study and that of Rebecca *et al*. (44.5% and 43.1%, respectively) [9]. However, the results from both studies were lower than Pegelow *et al*.'s 1997 study conducted in Sukaraja village, Bekasi, which reported a *Blastocystis* infection rate of 60% in elementary school children [3]. Other studies in the Melaka development area of Malaysia, Colombia, and the Thai-Myanmar border reported similar prevalence rates of *Blastocystis* infection among children aged 1–12 years old of 45.8% [10], 45% [11], and 37.2% [7], respectively.

These studies show that the prevalence rate of *Blastocystis* infection in the last ten years has remained constant in developing countries, ranging from 30% to 50%, while in developed countries it ranges from 1.5% to 10% [5]. These differences could be because the infection is not well recognized and understood, there have been no changes in the local environment and personal or community behaviour, or the source of infection is not recognized or identified. Based on age group, a higher prevalence rate of 66.6% (46 of 69) was found among children aged 6–9 years old than of10–13 years old; which was 33.3% (23 of 69). This findingcompared to children aged 10–13 years old differs from a study by Ashford *et al*. [12], in Papua New Guinea where the prevalence rate among young adults was higher than among the younger children.

The subtype analysis of *Blastocystis* in this study showed that ST3 was the most dominant subtype. It had 63.8% overall prevalence rate, followed by ST1 (27.5%) and ST2 (7.2%). These three subtypes were found in both symptomatic and asymptomatic groups. In the symptomatic group, four STs were identified; the most dominant subtype was ST3 (72.2%), followed by ST1 (22.2%), ST2 (2.8%), and ST4 (2.8%). ST1 and ST2 were found more frequently among the asymptomatic group ST3, which showed the reverse phenomenon. Another interesting finding was that ST4 was only found in one student who presented with diarrhea. The findings showing the dominance of ST3 are similar to most previous studies in Europe and Asia, such as Jamtemtor's study in Thailand that reported ST3 as the most dominant subtype (57.1%), followed by ST1 (21.4%), ST7 (17.9%) and ST6 (3.6%) [13]. Similarly, Wong *et al*.'s study in Singapore found ST3 to be the most dominant subtype (78%), followed by ST1 (22%) [14]. Boondit *et al*.'s study also reported ST3 as the most dominant subtype (76%), followed by ST1 (20%) [15]. Meloni *et al*. in Italy found the following ST distribution: ST3 (47.1%), ST2 (20.6%), ST4 (17.7%), ST1 (8.8%), and ST7 and ST8 (2.9%) [16]. A study by Dogruman *et al*. reported similar results, with ST3 being the most dominant in the symptomatic (59.3%) and asymptomatic groups (48.5%), followed by ST2 with 15.3% and 33.3% and ST1 with 20.3% and 15.2%. Similar subtype prevalence rates were also reported by Ozyurt *et al*. in Turkey and other countries such as China, Germany, Japan, and Denmark [17].

A different result was reported in a study by Awatif *et al*. in Libya where ST1 was the most dominant subtype in outpatients (51.1%), followed by ST2 (24.4%) and ST3 (17.8%) [4]. An alternate result was also reported in a study by Dominguez et al. in Spain where ST4 was the most dominant subtype at

94.1%, followed by ST1 (2%) and ST2 (3.9%) [18]. Souppart *et al*. stated that *Blastocystis* infection may not link to certain subtypes but to risk factors in infection transmission, including environmental factors (transmission route and source of contamination), parasite factors (pathogenic potential and zoonosis), and host factors (genotype, immunity, and age) [19]. A study by Hameed et al. examined *Blastocystis* protease activity in symptomatic and asymptomatic patients and found that *Blastocystis* protease was detected in 94.4% of patients; symptomatic patients expressed high protease levels in *Blastocystis* ST3 combined with 32 kDa low molecular weight protein exhibit virulence factor that is responsible for protein degradation that enables this parasite to evade the host immune system or modulation by degradation of host immune molecules [20].

4. Conclusion

A high prevalence of *Blastocystis* infection with four subtypes was found among primary schoolchildren in South Jakarta; ST3 was the most dominant and significantly associated with clinical symptoms.

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