



# Molecular Detection and Subtyping of Human *Blastocystis* and the Clinical Implications: Comparisons between Diarrheal and Non-diarrheal Groups in Korean Populations

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**Abstract:** *Blastocystis* has recently been recognized as the most common eukaryotic microbe of the human gut. We investigated the prevalence of *Blastocystis* and their subtypes in diarrheal and non-diarrheal groups and the associated clinical parameters. A total of 324 stool samples were obtained from 196 diarrheal and 128 non-diarrheal subjects. *Blastocystis* subtypes were determined by sequencing the small subunit ribosomal DNA (SSU rRNA) gene. Demographic, clinical and laboratory data were collected and analyzed by diarrhea and *Blastocystis* status. The overall rate of *Blastocystis* positivity was 9.0% (29/324) but was significantly higher in the non-diarrheal group (18.0% vs. 3.1%,  $P < 0.0001$ ). Of the 6 *Blastocystis*-positive diarrheal patients, 3 (50.0%), none (0.0%), 2 (33.3%), and 1 (16.7%) were infected with subtypes ST1, ST2, ST3, and multiple subtypes, respectively. Of the 23 *Blastocystis*-positive non-diarrheal patients, 4 (17.4%), 1 (4.3%), and 18 (78.3%) were infected with subtypes ST1, ST2, and ST3, respectively. *Blastocystis* was less common in the diarrheal than the non-diarrheal group (odds ratio, 0.144; 95% confidence interval, 0.057–0.365,  $P < 0.001$ ). Of the 3 subtypes, ST3 was more frequently observed in the non-diarrheal than diarrheal group (78.3% vs. 33.3%,  $P = 0.0341$ ). Collectively, *Blastocystis* was found in both the diarrheal and non-diarrheal groups and ST3 was the most common subtype in Korea.

**Key words:** *Blastocystis*, subtype, diarrhea, Korea

*Blastocystis* has recently been recognized as the most prevalent eukaryotic microbe in the human gut [1], occurring worldwide in both healthy and symptomatic humans and other animals. *Blastocystis* is thought to be transmitted via the fecal–oral route and in cyst form [2]. Recently, PCR-based approaches using feces directly or after culture of fecal specimens have been widely used to diagnose *Blastocystis* infection [3]. Based on small subunit ribosomal DNA (SSU rRNA) gene analysis, this genus comprises at least 17 subtypes (STs) [4]. In human, ST1–ST4 probably account for more than 90% of carriage; the other subtypes are ST5–ST9 [5]. Although *Blastocystis* is of great scientific interest, neither its biology nor pathophys-

iology has been well-explored. Since no direct evidence indicates that *Blastocystis* causes diarrhea, this point also remains controversial. Only 3 Korean studies on *Blastocystis* have been published [6–8], all in animals. Here, we investigated the prevalence of *Blastocystis* and its subtypes, and the associated clinical parameters, in diarrheal and asymptomatic Korean groups.

A total of 324 stool samples were obtained from 196 diarrheal and 128 non-diarrheal subjects who underwent general checkups at Chonnam National University Hospital and Chonnam National University Hwasun Hospital, from February 2016 to October 2018. Fecal samples were collected in accordance with the guidelines of, and with the approval of, the Institutional Review Board of Chonnam National University Hospital (approval no. IRB CNUH-2015-052). We recorded age, sex, white blood cell count (WBC,  $\times 10^3/\mu\text{l}$ ), and differential counts, red blood cell count (RBC,  $\times 10^6/\mu\text{l}$ ), hemoglobin (Hgb, g/dl), platelet count (PLT), total protein (g/dl), albumin (g/dl), alkaline phosphatase (U/L), AST (U/L), ALT (U/L),

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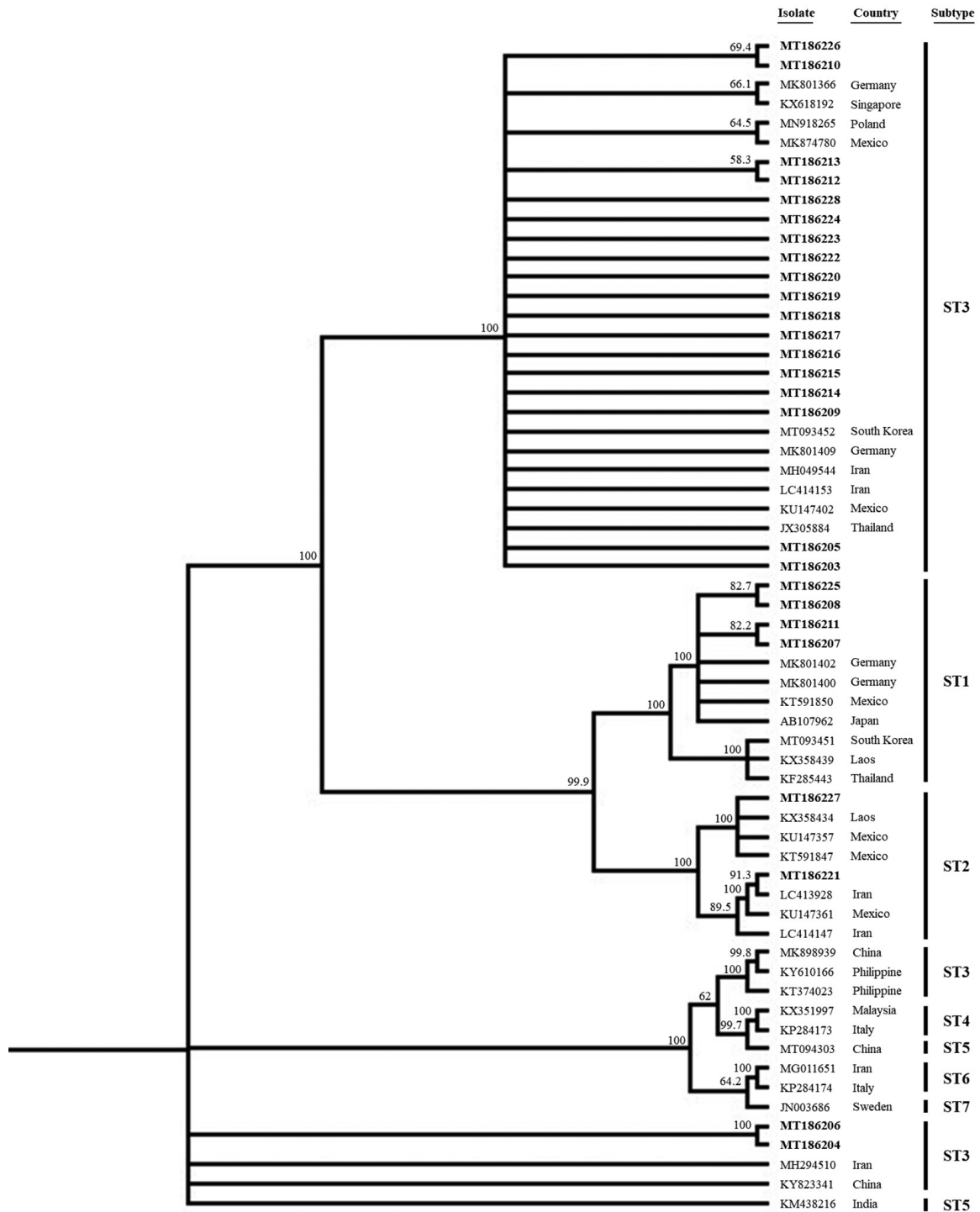
BUN (mg/dl), creatinine (mg/dl), lactate dehydrogenase (U/L), CRP (mg/dl), and stool occult blood test data. If necessary, stool culture or multiplex PCR panel evaluation was scheduled to determine pathogens causative of diarrhea. Laboratory findings and stool culture data were analyzed by diarrhea and *Blastocystis* status. DNA was extracted using the Cica Geneus® DNA Prep Kit (Kanto Chemical, Tokyo, Japan) following the manufacturer's instructions. *Blastocystis* was detected based on the SSU rRNA gene using the Blast-505-532 (5'-GGA GGT AGT GAC AAT AAA TC-3') and Blast-998-1017 (5'-TGC TTT CGC ACT TGT TCA TC-3') primers [9]. Each tube contained 8.5 µl PCR primer solution (1 µl each of Blast-505-532 and Blast-998-1017 (each 25 pmol)), 36.5 µl distilled water, and 5 µl template DNA. All PCR amplifications were performed using

the TaKaRa PCR Thermal Cycler Dice Gradient (TaKaRa, Tokyo, Japan). Initial denaturation at 94°C for 3 min was followed by 30 cycles of 59°C for 30 sec and 72°C for 60 sec, and a final extension at 72°C for 5 min. The PCR products were analyzed by 1.5% (w/v) agarose gel electrophoresis with ethidium bromide staining and then sent to Macrogen (Seoul, Korea) for direct DNA sequencing. Phylogenetic analysis was performed by reference to database *Blastocystis* SSU rRNA genes, and a phylogenetic tree was constructed using Geneious Prime (Biomatters Ltd, Auckland, New Zealand). Phylogenetic inferences were derived using a pair-group method featuring arithmetic average clustering with 1,000 bootstrap replications. Student's t-test was used to compare continuous variables (age and laboratory parameters). The chi-squared or Fisher's exact

**Table 1.** Characteristic of laboratory findings according to the diarrheal symptoms

Clinical parameters <sup>a</sup>	Laboratory characteristics of		P-value
	Diarrheal group (n=196)	Healthy control (n=128)	
Age (yr)	60.8±17.78	62.6±11.37	0.318
WBC count (x10 <sup>3</sup> /µl)	9±5.61	5.6±1.43	<0.001
RBC count (x10 <sup>6</sup> /µl)	3.6±0.66	4.8±0.43	<0.001
Hemoglobin (g/dl)	10.9±2.22	14.1±1.33	<0.001
Platelet count (x10 <sup>3</sup> /µl)	219.6±136.82	252.3±54.3	0.13
Lymphocytes (%)	15.8±11.27	34.9±8.01	<0.001
Monocytes (%)	9.3±7.54	6.8±2.18	0.034
Neutrophils (%)	73.1±14.94	66.1±73.8	0.224
Eosinophils (%)	1.9±2.21	2.8±2.69	0.015
Basophils (%)	0.4±0.38	0.7±0.27	<0.001
Total protein (g/dL)	6±0.99	7.5±0.48	<0.001
Albumin (g/dL)	3.3±0.75	4.4±0.3	<0.001
ALP (U/L)	114.4±134.06	75.7±22.59	0.058
AST (U/L)	36.1±38.3	29±12.01	0.067
ALT (U/L)	28.4±29.73	27.7±18.33	0.829
Total Bilirubin (mg/dL)	1.2±2.11	0.8±0.33	0.348
BUN (mg/dl)	17.6±14.35	12.1±4.89	0.011
Creatinine (mg/dl)	1.2±3.36	1.3±3.51	0.812
Lactate dehydrogenase (U/L)	618.9±450.65	141.5±94.76	<0.001
r-GTP (IU/L)	109±147.21	31.5±28.56	<0.001
CRP (mg/dl)	7.8±7.69	0.1±0.13	<0.001
Sex (male)	116/196 (59.2)	54/128 (42.2)	0.003
Presence of abdominal pain No. (%)	92/196 (46.9)	4/128 (3.1)	<0.001
Positive for stool occult blood No. (%)	9/23 (39.1)	0/86 (0.0)	<0.001
Positive for <i>Blastocystis</i> , No. (%)	6/196 (3.1)	23/128 (18.0)	<0.001
<i>Blastocystis</i> subtype No. (%)			
ST1	3/6 (50.0)	4/23 (17.4)	0.0964
ST2	0/6 (0.0)	1/23 (4.3)	
ST3	2/6 (33.3)	18/23 (78.3)	0.0341
Multi band	1/6 (16.7)	0/23 (0.0)	

<sup>a</sup>Clinical parameters, except for Sex, Stool occult blood, *Blastocystis*/subtype prevalence, were expressed with Mean±SD.



**Fig. 1.** Phylogenetic tree of 26 sequences of *Blastocystis* SSU rRNA gene compared to database *Blastocystis* SSU rRNA genes. All of sequences of this study were enrolled to GenBank database (GenBank accession no. MT186203-MT186218), which were indicated in bold.

test was employed to determine the distributions of categorical variables (sex and the statuses of stool occult blood, diarrhea, and *Blastocystis*). The likelihood-ratio chi-squared test was employed to calculate odds ratios (ORs) for *Blastocystis* positivity by subtype. All statistical analyses were performed using SPSS ver. 25.0 software (SPSS Inc., Chicago, Illinois, USA). A *P*-value < 0.05 was considered to indicate significance.

Of the 324 samples, 29 (9.0%) including 6 diarrheal and 23 non-diarrheal samples were positive for *Blastocystis*, with a significant difference between the 2 groups (*P* < 0.001) (Table 1). Thus, *Blastocystis* may not necessarily cause diarrhea. *Blastocystis* has previously been found in both symptomatic and asymptomatic patients [10-12]. It has been suggested that intra-subtype variation at the SSU rRNA gene level might affect the presenting symptoms of patients with identical *Blastocystis* sub-

types [13]. We found subtypes ST1, ST2, ST3, and multiple infections in 3 (50.0%), 0 (0.0%), 2 (33.3%), and 1 (16.7%) patient in the diarrheal group and in 4 (17.4%), 1 (4.3%), 18 (78.3%), and 0 (0.0%) patient the non-diarrheal group. Of the 3 subtypes, ST3 was more common in the non-diarrheal group (78.3 vs. 33.3%, *P* = 0.0341). ST1 seemed to be frequently found in diarrheal group compared to non-diarrheal group, but no statistical significance (diarrheal group vs. non-diarrheal group, 3/6 vs. 4/23, *P* = 0.0964). Further study may be necessary to determine potential correlation of certain subtypes and symptoms. We did not detect ST4, in agreement with previous studies suggesting that this ST was rare in Asia and the Middle East [14,15]. When we analyzed the distribution of STs, a total of 26 sequenced samples (GenBank accession no. MT186203-MT186218 enrolled in this study) were closely related to the se-

**Table 2.** Characteristic of laboratory findings according to the presence of *Blastocystis*

Clinical parameters <sup>a</sup>	Laboratory characteristics		<i>P</i> -value
	<i>Blastocystis</i> -negative (n=295)	<i>Blastocystis</i> -positive (n=29)	
Age (yr)	61.4 ± 15.94	62.6 ± 11.48	0.692
WBC count (x10 <sup>3</sup> /μl)	8.5 ± 5.29	6.5 ± 4.75	0.234
RBC count (x10 <sup>6</sup> /μl)	3.8 ± 0.76	4.1 ± 0.9	0.301
Hemoglobin (g/dl)	11.9 ± 2.48	12.7 ± 2.2	0.147
Platelet count (x10 <sup>3</sup> /μl)	226.6 ± 128.34	197.7 ± 82.64	0.482
Lymphocytes (%)	19.1 ± 13.11	23.9 ± 11.13	0.254
Monocytes (%)	8.8 ± 6.99	8.9 ± 5.8	0.965
Neutrophils (%)	72.1 ± 34.9	64.4 ± 13.94	0.488
Eosinophils (%)	2 ± 2.35	2.2 ± 2.1	0.808
Basophils (%)	0.4 ± 0.39	0.5 ± 0.38	0.495
Total protein (g/dl)	6.3 ± 1.09	6.5 ± 1.07	0.606
Albumin (g/dl)	3.5 ± 0.82	3.9 ± 0.56	0.083
ALP (U/L)	107.5 ± 121.95	64.9 ± 14.12	0.357
AST (U/L)	34.3 ± 32.85	24.8 ± 7.42	0.177
ALT (U/L)	28.5 ± 27.09	23.5 ± 10.74	0.387
Total Bilirubin (mg/dl)	1.1 ± 1.93	0.7 ± 0.2	0.515
BUN (mg/dl)	16.7 ± 13.25	13 ± 12.89	0.299
Creatinine (mg/dl)	1.1 ± 2.82	3.6 ± 7.37	0.001
Lactate dehydrogenase (U/L)	499.6 ± 446.27	225.9 ± 199.58	0.109
r-GTP (IU/L)	70.8 ± 112.65	25.6 ± 17.51	0.137
CRP (mg/dl)	6.8 ± 7.69	5.5 ± 5.94	0.613
Sex male No. (%)	159/295 (53.9)	11/29 (37.9%)	0.12
Presence of abdominal pain No. (%)	93/295 (31.5)	3/29 (10.3%)	0.018
Positive for stool occult blood No. (%)	9/95 (9.5)	0/14 (0.0%)	0.601
Positive for Diarrhea No. (%)	190/295 (64.4)	6/29 (20.7%)	< 0.001
Other pathogens No. (%)	52/295 (17.6) <sup>b</sup>	2/29 (6.9%) <sup>c</sup>	

<sup>a</sup>Clinical parameters, except for Sex, Stool occult blood, Diarrhea, Other pathogens, were expressed with Mean ± SD.

<sup>b</sup>*Clostridium difficile* and/or the other pathogens(23), *Campylobacter* spp. and/or the other pathogens(14), *Salmonella* spp. (9), *Citrobacter freundii* (1), *Cryptosporidium* spp. (1), *Enterococcus faecalis* (1), ETEC LT/ST,STEC stx1/sb2 (1), Norovirus GI/GII (1), and *Shigella* spp. (1).

<sup>c</sup>*Campylobacter* spp. (1) and *Giardia* spp. (1).

quences of each known human STs (Fig. 1). All but 2 ST3 sequences clustered together with sequences from the other countries such as Germany (MK801366, MK801409), Singapore (KX618192), Poland (MN918265), Mexico (MK874780, KU147402), South Korea (MT093452), Iran (MH049544, LC414153) and Thailand (JX305884), respectively. Notably, 2 ST3 sequences (MT186204, MT186206) from the diarrheal group were somewhat distant from the others; ST3 thus exhibited intra-genetic variation. All sequences lay distant from those of ST4 (KX351997, KP284173), ST5 (MT094303, KM438216), ST6 (MG011651, KP284174), and ST7 (JN003686), respectively. We also analyzed several laboratory parameters by *Blastocystis* presence or symptoms. The white and red blood cell counts, hemoglobin level, percentages of lymphocytes, monocytes, eosinophils, and basophils, and the levels of total protein, albumin, BUN, lactate dehydrogenase, gamma-glutamyl transpeptidase, and C-reactive protein, and the rate of positive results on stool occult blood tests differed significantly between the diarrheal and non-diarrheal groups. However, only the creatinine level differed significantly between the *Blastocystis*-positive and -negative groups (3.6 vs. 1.1 mg/dl,  $P=0.001$ ) (Table 2). Of the 196 diarrheal patients, 54 exhibited other causative pathogens (*Clostroides difficile* with or without other pathogens (23), *Campylobacter* spp. with or without other pathogens (15), *Salmonella* spp. (9), *Citrobacter freundii* (1), *Cryptosporidium* spp. (1), *Enterococcus faecalis* (1), ETEC LT/ST, STEC stx1/sb2 (1), Norovirus GI/GII (1), *Giardia* spp. (1) and *Shigella* spp. (1)). Of the 29 *Blastocystis*-positive patients, all but 2 (one with *Campylobacter* sp. and one with *Giardia* sp.) were negative for other possibly causative pathogens. Overall, our data support the suggestion that *Blastocystis* may not be pathogenic.

This is the first report of *Blastocystis* infections in Koreans. *Blastocystis* was found in both the diarrheal and non-diarrheal groups, but the subtype prevalence differed between the groups. Any role played by *Blastocystis* in human health and disease should be explored further.

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design, data collection and analysis decision to publish or preparation of the manuscript.

## CONFLICT OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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