Annals of Parasitology 2016, 62(3), 193–200
doi: 10.17420/ap6203.53

Original papers

Subtype analysis of *Blastocystis* sp. isolates from asymptomatic individuals in an urban community in the Philippines

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ABSTRACT. *Blastocystis* sp. is a commonly reported enteric protistan parasite in faecal specimens with a worldwide distribution afflicting both humans and a wide range of animals. The aim of this study is to characterize the subtypes (STs) of *Blastocystis* sp. isolates from asymptomatic individuals in an urban community in Pateros, Metro Manila, Philippines. The 600-bp small subunit ribosomal RNA (SSU rRNA) barcoding region of *Blastocystis* sp. isolates was amplified and sequenced using the primers RD5 and BhRDr. Subtypes were identified by uploading the sequences onto the Basic Local Alignment and Search Tool (BLAST) websites, the *Blastocystis* Subtype (18S) and Sequence Typing (MLST) Database and by construction of a phylogenetic tree. Twenty-nine (29) out of 35 individuals were detected positive for *Blastocystis* sp. ST3 is the most common among the three STs detected (65.5%), followed by ST1 (31.0%) and ST4 (3.44%). This study showed that DNA barcoding can serve as a helpful tool to investigate the diversity of *Blastocystis* sp. in the Philippines.

Key words: DNA barcoding, SSU rRNA gene, *Blastocystis*, subtypes, Philippines

Introduction

Blastocystis sp. is an enteric single-celled, anaerobic, protistan parasite of humans and animals with enigmatic and controversial pathogenicity, and clinical relevance [1]. It is the most prevalent non-fungal micro-eukaryote reported in the human intestinal tract and faecal samples [2–4]. It has infected the large intestine of more than one billion people [3] and has a far higher prevalence than *Giardia* sp., *Entamoeba histolytica* and *Cryptosporidium* sp. [5]. Transmission is mainly via the faecal-oral route and there is evidence of its zoonotic nature [6–8]. The cyst stage, being water-resistant, is its transmissible stage [9] and the amoeboid stage has been linked with pathogenicity [2].

Seventeen *Blastocystis* sp. subtypes (STs) have been identified based on full sequences of its small subunit rRNA (SSU rRNA) gene [10]. However, these STs can also be distinguished using a 600-bp barcoding region at the 5'-end of the same gene [11]. *Blastocystis* sp. cells from each ST are morphologically indistinguishable from the cells of other STs, but possess remarkable sequence divergences. Stenzel and Boreham [1] have stated that existing knowledge of *Blastocystis* sp. and the putative disease it causes is not yet sufficient to elucidate the relevance of the parasite in humans. Hence, tremendous efforts using molecular studies and surveys have been made to fully understand the controversial biology of this parasite and its ramifications on public health [2].

Blastocystis sp. has been associated with irritable bowel syndrome [5,12], abdominal pains [13–15], diarrhoea [14,15], and even haematological malignancy [16]. In particular, ST4 has

been associated with acute diarrhoea in patients from Denmark [17], ST1 and ST3 with irritable bowel syndrome in patients from Mexico [18], and ST2 with a patient with recurring gastrointestinal and urticarial symptoms from Europe [19]. However, direct links between disease and presence of the parasite are yet to be described [20]. In the Philippines, *Blastocystis* genomic studies based on full SSU rRNA gene sequences have commenced on animal and human subjects [6,21,22] as well as on wastewater isolates [23]. Recently, *Blastocystis* sp. ST identification was also performed in backyard farm animals in the Philippines using the 600-bp barcoding region [24].

The aim of the present study was to supplement existing data on *Blastocystis* sp. ST diversity in the Philippines by identifying the STs of isolates from residents of an urban community in Pateros, Metro Manila, Philippines. This is the first study on ST identification of *Blastocystis* sp. human isolates from a Philippine community using the barcoding primers [11].

Materials and Methods

Sample collection. Faecal samples were randomly collected from 35 residents of Pateros, Metro Manila, Philippines. This urban community is located southeast of Metropolitan Manila (Fig. 1). The specimens were collected from permanent residents in the municipality who had not taken metronidazole or antidiarrheal medications two weeks prior to sample collection. Gross macroscopic examination of faecal samples was performed to check for consistency and presence of adult helminths. Written informed consent was obtained from participants, parents, or guardians if unable to give consent before starting the study. This study protocol was approved by the Ethics Review Committee of the College of Public Health, University of the Philippines, Manila, Philippines.

Isolation and cultivation of *Blastocystis*. Approximately 50 mg faecal specimens were aseptically inoculated in diphasic medium supplemented with 10% heat-inactivated horse serum and 1% penicillin-streptomycin antibiotics [6]. Cultures were maintained at 37°C and sub-cultured every 3–4 days. Positive cultures were passaged into fresh medium two times before being subjected to DNA extraction.

DNA extraction. DNA was extracted from *Blastocystis* sp. cells collected from 3-4 day old

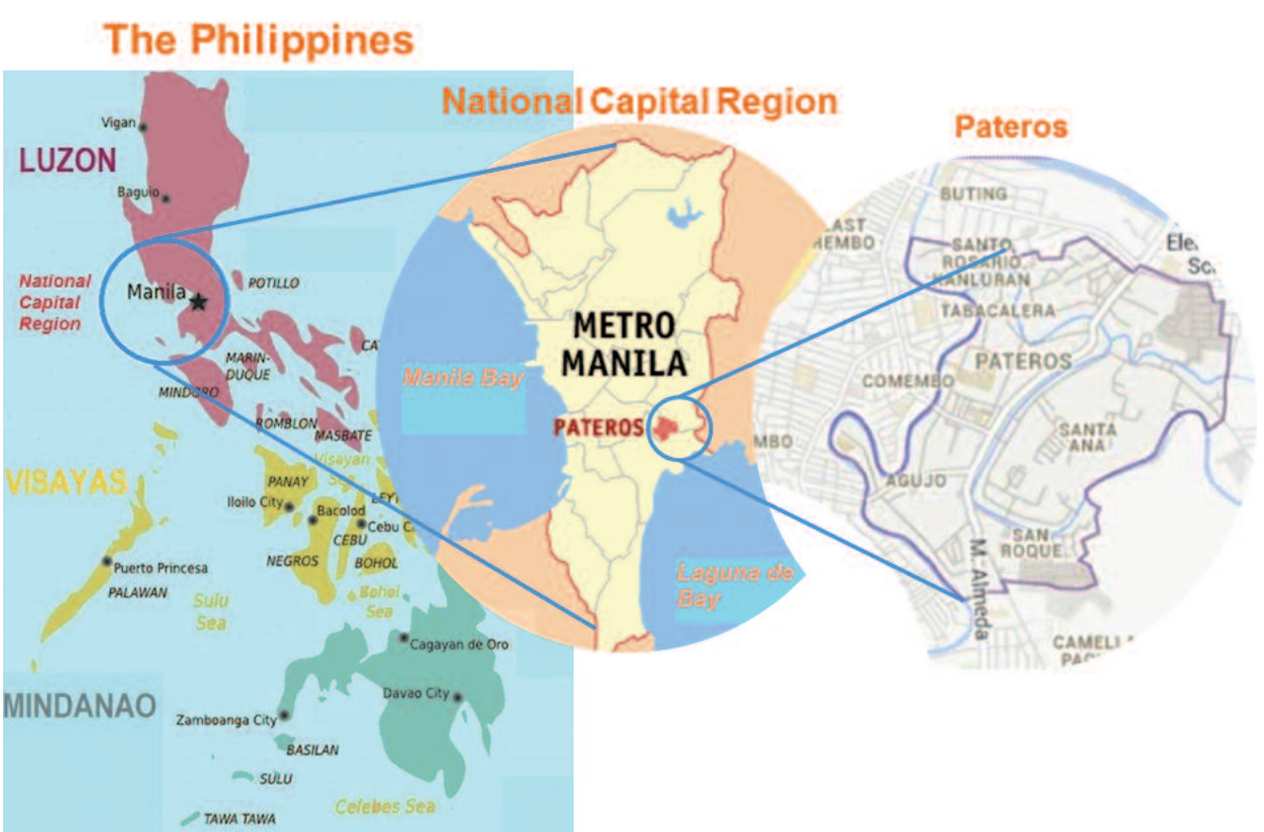

Fig. 1. Map of the Philippines showing the location of the sampling site

Table 1. *Blastocystis* sp. subtypes (STs) and most similar sequences in GenBank of samples identified using the Basic Local Alignment and Search Tool (BLAST) and *Blastocystis* Subtype (18S) and Sequence Typing (MLST) Database websites

Sample (GenBank accession no.)	Percent identity	Percent coverage	Most similar GenBank sequence
5H2b (KP408435)	99%	99%	*Blastocystis* sp. BLERC01, Turkey, human, JX448397 (ST3)
6H1F (KP408436)	100%	99%	*Blastocystis* sp. BLERC01, Turkey, human, JX448397 (ST3)
10H2 (KP408437)	99%	99%	*Blastocystis* sp. BLERC01, Turkey, human, JX448397 (ST3)
14H2b (KP408438)	99%	99%	*Blastocystis* sp. BLERC01, Turkey, human, JX448397 (ST3)
15H1F (KP408439)	99%	99%	*Blastocystis* sp. BLERC01, Turkey, human, JX448397 (ST3)
16H1Fb (KP408440)	99%	99%	*Blastocystis* sp. MKJ04-19, Japan, *Dendrolagus goodfellowi* (Goodfellow's tree kangaroo), EU427516 (ST4)
17H2 (KP408441)	99%	99%	*Blastocystis* sp. JEB-2010 isolate E3, Philippines, wastewater, GU992419 (ST1)
18H2b (KP408442)	100%	99%	*Blastocystis* sp. BLERC01, Turkey, human, JX448397 (ST3)
19H2 (KP408443)	98%	99%	*Blastocystis* sp. BLERC01, Turkey, human, JX448397 (ST3)
20H2b (KP408444)	100%	99%	*Blastocystis* sp. BLERC01, Turkey, human, JX448397 (ST3)
21H2b (KP408445)	99%	99%	*Blastocystis* sp. CK86-1, Japan, chicken, AB070993 (ST1)
23H1F (KP408446)	99%	99%	*Blastocystis* sp. BLERC01, Turkey, human, JX448397 (ST3)
24H2b (KP408447)	99%	99%	*Blastocystis* sp. BLERC01, Turkey, human, JX448397 (ST3)
25H2 (KP408448)	99%	99%	*Blastocystis* sp. BLERC01, Turkey, human, JX448397 (ST3)
26H2 (KP408449)	100%	99%	*Blastocystis* sp. BLERC01, Turkey, human, JX448397 (ST3)
27H2 (K408450)	100%	99%	*Blastocystis* sp. BLERC01, Turkey, human, JX448397 (ST3)
28H2b (KP408451)	99%	99%	*Blastocystis* sp. M9, Philippines, monkey, EU445490 (ST1)
29H1F (KP408452)	99%	99%	*Blastocystis* sp. OSU6, USA, human, EU679349 (ST1)
30H2b (KP408453)	99%	100%	*Blastocystis* sp. BLERC01, Turkey, human, JX448397 (ST3)
H0927 (KT374017)	98%	99%	*Blastocystis* sp. CK86-1, Japan, chicken, AB070993 (ST1)
H1061 (KT374018)	99%	100%	*Blastocystis* sp. M2, Philippines, Philippine macaque, EU445488 (ST1)
H1104 (KT374019)	98%	99%	*Blastocystis* sp. JEB-2010 isolate E3, Philippines, wastewater, GU992419 (ST1)
H0838 (KT374020)	99%	99%	*Blastocystis* sp. M2, Philippines, Philippine macaque, EU445488 (ST1)
0850 (KT374021)	99%	99%	*Blastocystis* sp. CK86-1, Japan, chicken, AB070993 (ST1)
H0552 (KT374022)	99%	100%	*Blastocystis* sp. BLERC01, Turkey, human, JX448397 (ST3)
H0585 (KT374023)	99%	100%	*Blastocystis* sp. BLERC01, Turkey, human, JX448397 (ST3)
H0593 (KT374024)	98%	99%	*Blastocystis hominis* isolate Ah10 KP244347, Iran, human, KP244347 (ST3)
H0554 KT374025)	100%	99%	*Blastocystis hominis* isolate DSS60, Senegal, human, KF848577 (ST3)
H0657 (KT374026)	100%	99%	*Blastocystis* sp. BLERC01, Turkey, human, JX448397 (ST3)

cultures using the Chelex method [25]. Briefly, 1.5 ml of the culture was centrifuged at 10,000 rpm for two minutes to harvest the cells. The pellets were washed twice with 1 ml of phosphate-buffered saline before re-suspension in 100 µl sterile distilled water and 200 µl of 5% Chelex-100. The resulting suspension was incubated at 56°C for 30 minutes, vortexed briefly, and boiled for eight minutes using water bath. Suspensions were then centrifuged at 13,000 rpm for two minutes. The supernatant containing the DNA was transferred to a new microfuge tube, and kept frozen until used.

Polymerase chain reaction (PCR). The 600-bp barcoding region of the SSU rRNA gene of *Blastocystis* sp. was amplified using the primers RD5 and BhRDr [11]. Amplification was performed using 30 cycles consisting of one minute each of 94°C, 59°C, and 72°C with an additional two-minute final extension at 72°C. Results were visualized in 1.5% agarose gels stained with 5.0 ng/ml ethidium bromide and viewed under a UV transilluminator. PCR products that yielded the 600-bp target were sent to Macrogen Inc. in Seoul, South Korea for purification and sequencing.

Phylogenetic analysis. Reverse and forward sequences were aligned manually using the program, BioEdit v.7.0 [26]. These sequences were uploaded to the Basic Local Alignment and Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov /Blast.cgi) to determine the most similar sequence of each isolate, and to the *Blastocystis* Subtype (SSU rRNA) and Sequence Typing (MLST) Database (http://pubmlst.org/blastocystis) to determine their ST. Sequences from this study, along with reference sequences for ST1-ST10, ST15, ST16, ST17, and *Proteromonas lacertae* SSU rRNA (GenBank accession no. U37108) as an outgroup, were aligned using the ClustalW feature of BioEdit v. 7.0 [26]. The assemblies were then manually aligned via ocular inspection to remove ambiguous sequences in the alignment.

The general time reversible (GTR+G) model was determined to be the best model for use after comparing the likelihoods (–ln L) of all possible models using the jModelTest program [27]. Neighbor-joining (NJ) and Bayesian inference (BI) phylogenetic trees were constructed using PAUP*v.4.0b10 [28] and MrBayes v.3.1 [29], respectively. The maximum likelihood (ML) phylogenetic tree was constructed by uploading the aligned sequences onto the www.phyml.org website [30]. Clusters with branches of >50% bootstrap support (NJ and ML) and >0.70 posterior probability (BI) were considered valid for this study.

Results

Twenty-nine (29) out of 35 asymptomatic individuals living in Pateros, Metro Manila were found to be positive for *Blastocystis* sp. using culture and PCR. The DNA sequences generated from the barcoding region of SSU rRNA gene were 99–100% similar to *Blastocystis* sp. sequences from GenBank (Table 1). The results of the phylogenetic analysis and the subtyping using the website showed that 19 of the 29 (65.5%) *Blastocystis* sp. isolates were ST3, 9 (31.0%) were ST1, and 1 (3.44%) was ST4. All sample isolate sequences except for 28H2b clustered with their respective similar ST sequences in the phylogenetic trees (Fig. 2). The sequences were stored in GenBank under the accession nos. KP408435 to KP408453 and KT374017 to KT374026.

Discussion

Blastocystis sp. is frequently encountered in parasitological surveys in the Philippines with a prevalence as high as 40.7% in children living in Metro Manila [31] and 36.8% in patients from the Philippine General Hospital and the College of Public Health in the University of the Philippines-Manila [22]. In this study, the prevalence of *Blastocystis* sp. was higher than in previous studies at 82.9% (29/35) of asymptomatic residents of Pateros. The prevalence of *Blastocystis* sp. can be as high as 100%, as shown in a survey conducted in children from Senegal [4]. Belleza et al. [32] found an association between dog ownership and *Blastocystis* sp. infection in a previous study in residents of Pateros. In this study, all 35 individuals were dog owners. However, samples were not obtained from the pet dogs of these residents. Thus, proof of possible zoonotic transmission between pet and owner is still lacking. The high prevalence may be due to other factors such as poor hygiene practices.

This study shows the utility of molecular subtyping using DNA barcoding of *Blastocystis* sp. [11]. Previous subtyping studies on Philippine isolates made use of full SSU rRNA gene sequences [6,23]. The use of barcode sequences has several advantages over the entire SSU rRNA gene sequence. These short barcode sequences can be used in publicly available sequence databases that can render consensus ST nomenclature and locus and allele assignments per barcode sequence query much more conveniently than using full SSU sequences [3,33]. Another advantage of using the DNA barcoding method of Scicluna et al. [11] is that the barcode sequences of the primers cover all polymorphic positions, ensuring that no phylogenetic signal information is lost. Scicluna et al. [11] have demonstrated that the barcode sequence generated from only either forward or

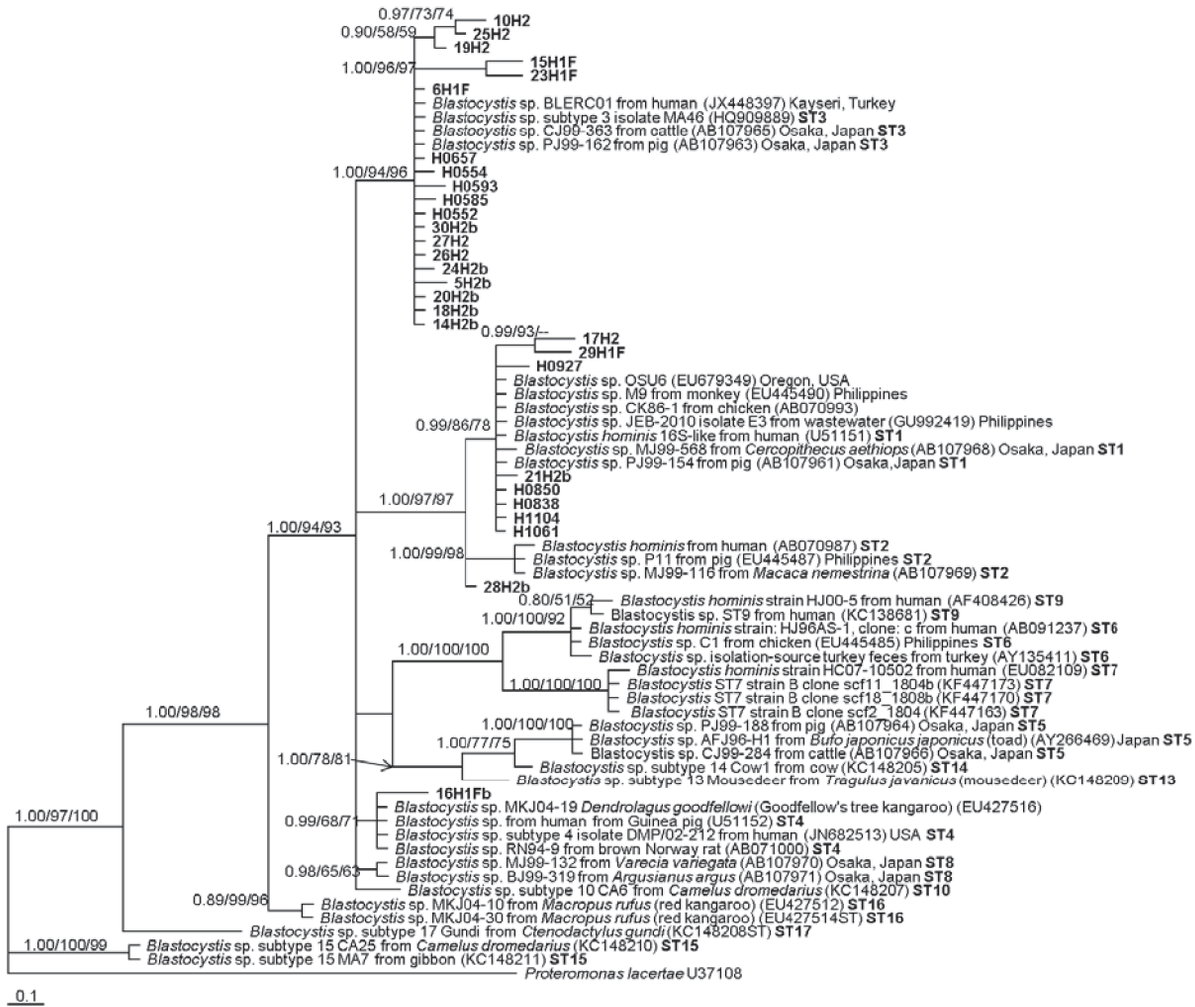

Fig. 2. Consensus phylogenetic tree constructed from 419 unambiguously aligned nucleotide basepairs of the 29 samples and 40 *Blastocystis* sp. 18S SSU rRNA reference sequences. The consensus tree is based on the Bayesian Inference (BI) tree. Values above the branches represent bootstrap support or posterior probability for that particular cluster in the order of Bayesian inference value/maximum-likelihood bootstrap support/neighbor-joining bootstrap support. The scale bar at the bottom represents one nucleotide basepair change for every position composed of 10 nucleotides.

reverse primer was sufficient for barcoding. In this study, only forward sequences were used for the 16H1F, 15H1F, 16H1Fb, 23H1F, and 29H1F since the reverse sequences had mixed chromatogram peaks possibly from amplification of contaminants.

All sequences obtained in this study clustered with their respective STs except for 28H2b. However, this sample isolate sequence is still declared as ST1 based on both BLAST and pubmlst.org subtyping results. The sample isolate sequence was 99% similar to *Blastocystis* sp. M9 ST1 (Genbank accession no. EU445490) based on BLAST results. The reduced number of aligned unambiguous nucleotide basepair (bp) positions used in this study (463 bp) compared to when the entire SSU rRNA gene is used may be the reason why the 28H2b sequence clustered separately from other ST1 sequences but still within the ST1/ST2 cluster. Moreover, there may be intra-ST variation in ST1. Previous phylogenetic studies involving *Blastocystis*, including that of Noël et al. [34] and Scicluna et al. [11], had encountered similar instances of topology variability, especially within subgroups. According to current consensus, the presence of a < 3% sequence difference between complete SSU rRNA sequences indicates variation within an ST, while a difference of at least 5% indicates a new ST [10]. In this case, only the 600-bp barcoding region was sequenced and not all representative ST1 and ST3 sequences were used. Thus, the case of 28H2b and other smaller clusters cannot yet be declared as being within ST

variations. Nevertheless, the use of the barcode region was sufficient in identifying the STs of the isolates.

ST1 and ST3 were the most common forms found in the residents of Pateros. Similarly, these STs are also common in patients from other countries [35–38], as well as in non-human primates [39] and livestock [10,38]. ST3 is usually the most common ST found in humans while ST1, ST2, and ST4 are the next most common [4,35,40,41]. In other Southeast Asian countries including Singapore [42], Thailand [43] and the Thai-Myanmar border [44], ST3 was also found to be the predominant form, followed by ST1. One exception was a survey of the residents of a home for girls in Bangkok, Thailand where ST3 was not detected; ST1 was the predominant ST followed by ST6 and ST2 [45]. Interestingly, one of the isolates (17H2) was 99% similar to *Blastocystis* sp. ST1 previously isolated from wastewater in the Philippines (JEB-2010, GenBank accession no. GU992419) [23]. The cyst form of *Blastocystis* sp. can be found in water samples [46–50] suggesting a high possibility of waterborne transmission. In particular, Lee et al. [46] found ST1 and ST4 *Blastocystis* sp. in both river water samples and livestock, as well as in the residents of a village in Nepal.

With such remarkable genetic heterogeneity, it is very likely that new *Blastocystis* STs will be discovered soon. Dwelling on the advantages and the utility of the barcode region, it is recommended to utilize the DNA barcoding method to detect potential new STs. Our findings encourage further large-scale *Blastocystis* molecular subtyping surveys in the Philippines to identify more STs present in the country. Future subtyping surveys involving human hosts should also include symptomatic individuals to further highlight the relationship between the STs and disease, and to further supplement the emerging consensus on *Blastocystis* pathogenicity.

Acknowledgements

This work was supported by a research grant from the Natural Sciences Research Institute, University of the Philippines, Diliman.

References

[1] Stenzel D., Boreham P. 1996. *Blastocystis hominis* revisited. *Clinical Microbiology Reviews* 9: 563-584.

[2] Tan K.S. 2008. New insights on classification, identification, and clinical relevance of *Blastocystis* spp. *Clinical Microbiology Reviews* 21: 639-665.

[3] Stensvold C.R. 2013. *Blastocystis*: genetic diversity and molecular methods for diagnosis and epidemiology. *Tropical Parasitology* 3: 26-34.

[4] El Safadi D., Gaayeb L., Meloni D., Cian A., Poirier P., Wawrzyniak I., Delbac F., Dabboussi F., Delhaes L., Seck M. 2014. Children of Senegal River Basin show the highest prevalence of *Blastocystis* sp. ever observed worldwide. *BMC Infectious Diseases* 14: 164. doi:10.1186/1471-2334-14-164.

[5] Meloni D., Sanciu G., Poirier P., El Alaoui H., Chabe M., Delhaes L., Dei-Cas E., Delbac F., Luigi Fiori P., Di Cave D., Viscogliosi E. 2011. Molecular subtyping of *Blastocystis* sp. isolates from symptomatic patients in Italy. *Parasitology Research* 109: 613-619.

[6] Rivera W.L. 2008. Phylogenetic analysis of *Blastocystis* isolates from animal and human hosts in the Philippines. *Veterinary Parasitology* 156: 178-182.

[7] Yoshikawa H., Wu Z., Pandey K., Pandey B.D., Sherchand J.B., Yanagi T., Kanbara H. 2009. Molecular characterization of *Blastocystis* isolates from children and rhesus monkeys in Kathmandu, Nepal. *Veterinary Parasitology* 160: 295-300.

[8] Noël C., Peyronnet C., Gerbod D., Edgcomb V.P., Delgado-Viscogliosi P., Sogin M.L., Capron M., Viscogliosi E., Zenner L. 2003. Phylogenetic analysis of *Blastocystis* isolates from different hosts based on the comparison of small-subunit rRNA gene sequences. *Molecular and Biochemical Parasitology* 126: 119-123.

[9] Suresh K., Smith H., Tan T. 2005. Viable *Blastocystis* cysts in Scottish and Malaysian sewage samples. *Applied and Environmental Microbiology* 71: 5619-5620.

[10] Alfellani M.A., Taner-Mulla D., Jacob A.S., Imeede C.A., Yoshikawa H., Stensvold C.R., Clark C.G. 2013. Genetic diversity of *Blastocystis* in livestock and zoo animals. *Protist* 164: 497-509.

[11] Scicluna S.M., Tawari B., Clark C.G. 2006. DNA barcoding of *Blastocystis*. *Protist* 157: 77-85.

[12] Yakoob J., Jafri W., Jafri N., Khan R., Islam M., Beg M.A., Zaman V. 2004. Irritable bowel syndrome: in search of an etiology: role of *Blastocystis hominis*. *American Journal of Tropical Medicine and Hygiene* 70: 383-385.

[13] Andiran N., Acikgoz Z.C., Turkay S., Andiran F. 2006. *Blastocystis hominis* – an emerging and imitating cause of acute abdomen in children. *Journal of Pediatric Surgery* 41: 1489-1491.

[14] Al-Fellani M.A., Khan A.H., Al-Gazoui R.M., Zaid M.K., Al-Ferjani M.A. 2007. Prevalence and clinical features of *Blastocystis hominis* infection among patients in Sebha, Libya. *Sultan Qaboos University Medical Journal* 7: 35-40.

[15] Jerez Puebla L., Millán I., Núñez Fernández F. 2014. Frequency of blastocystosis and its association with clinical symptoms in 2 years of surveillance at „Pedro Kourí" Institute. *Clinical Microbiology* 3: 178. doi:10.4172/2327-5073.1000178.

[16] Taşova Y., Sahin B., Koltaş S., Paydaş S. 2000. Clinical significance and frequency of *Blastocystis hominis* in Turkish patients with hematological malignancy. *Acta Medica Okayama* 54: 133-136.

[17] Stensvold C.R., Christiansen D.B., Olsen K.E.P., Nielsen H.V. 2011. *Blastocystis* sp. subtype 4 is common in Danish *Blastocystis*-positive patients presenting with acute diarrhea. *American Journal of Tropical Medicine and Hygiene* 84: 883-885.

[18] Jimenez-Gonzalez D.E., Martinez-Flores W.A., Reyes-Gordillo J., Ramirez-Miranda M.E., Arroyo-Escalante S., Romero-Valdovinos M., Stark D., Souza-Saldivar V., Martinez-Hernandez F., Flisser A. 2012. *Blastocystis* infection is associated with irritable bowel syndrome in a Mexican patient population. *Parasitology Research* 110: 1269-1275.

[19] Vogelberg C., Stensvold C.R., Monecke S., Ditzen A., Stopsack K., Heinrich-Gräfe U., Pöhlmann C. 2010. *Blastocystis* sp. subtype 2 detection during recurrence of gastrointestinal and urticarial symptoms. *Parasitology International* 59: 469-471.

[20] Chen T.L., Chan C.C., Chen H.P., Fung C.P., Lin C.P., Chan W.L., Liu C.Y. 2003. Clinical characteristics and endoscoic findings associated with *Blastocystis hominis* in healthy adults. *American Journal of Tropical Medicine and Hygiene* 69: 213-216.

[21] Rivera W.L., Tan M.A. 2005. Molecular characterization of *Blastocystis* isolates in the Philippines by riboprinting. *Parasitology Research* 96: 253-257.

[22] Tan M.A.V., Rivera W.L. 2009. Genotypic characterization of *Blastocystis* isolates in the Philippines. *Philippine Journal of Veterinary Medicine* 45: 86-94.

[23] Banaticla J.E., Rivera W.L. 2011. Detection and subtype identification of *Blastocystis* isolates from wastewater samples in the Philippines. *Journal of Water and Health* 9: 128-137.

[24] Adao D.E.V., Ducusin R.J.T., Padilla M.A., Rivera W.L. 2016. Molecular characterization of *Blastocystis* isolates infecting farm animals in Victoria and Pila, Laguna, Philippines. *The Philippine Agricultural Scientist* (in press).

[25] Walsh P.S., Metzger D.A., Higuchi R. 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques* 10: 506-513.

[26] Hall T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41: 95-98.

[27] Posada D. 2008. jModelTest: phylogenetic model averaging. *Molecular Biology and Evolution* 25: 1253-1256.

[28] Swofford D.L. 2003. PAUP*. Phylogenetic analysis using parsimony (* and other methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

[29] Ronquist F., Huelsenbeck J.P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572-1574.

[30] Guindon S., Dufayard J.F., Lefort V., Anisimova M., Hordijk W., Gascuel O. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology* 59: 307-321.

[31] Baldo E.T., Belizario V.Y., De Leon W.U., Kong H.-H., Chung D.-I. 2004. Infection status of intestinal parasites in children living in residential institutions in Metro Manila, the Philippines. *Korean Journal of Parasitology* 42: 67-70.

[32] Belleza M.L.B., Cadacio J.L.C., Borja M.P., Solon J.A.A., Padilla M.A., Tongol-Rivera P.N., Rivera W.L. 2015. Epidemiologic study of *Blastocystis* infection in an urban community in the Philippines. *Journal of Environmental and Public Health.* doi:10.1155/2015/894297.

[33] Stensvold C.R., Suresh G.K., Tan K.S., Thompson R., Traub R.J., Viscogliosi E., Yoshikawa H., Clark C.G. 2007. Terminology for *Blastocystis* subtypes – a consensus. *Trends in Parasitology* 23: 93-96.

[34] Noël C., Dufernez F., Gerbod D., Edgcomb V.P., Delgado-Viscogliosi P., Ho L.C., Singh M., Wintjens R., Sogin M.L., Capron M. 2005. Molecular phylogenies of *Blastocystis* isolates from different hosts: implications for genetic diversity, identification of species, and zoonosis. *Journal of Clinical Microbiology* 43: 348-355.

[35] Alfellani M.A., Stensvold C.R., Vidal-Lapiedra A., Onuoha E.S., Fagbenro-Beyioku A.F., Clark C.G. 2013. Variable geographic distribution of *Blastocystis* subtypes and its potential implications. *Acta Tropica* 126: 11-18.

[36] Forsell J., Granlund M., Stensvold C.R., Clark C.G., Clark G.C., Evengard B. 2012. Subtype analysis of *Blastocystis* isolates in Swedish patients. *European Journal of Clinical Microbiology Infectious Diseases* 31: 1689-1696.

[37] Li L.H., Zhou X.N., Du Z.W., Wang X.Z., Wang L.B., Jiang J. Y., Yoshikawa H., Steinmann P., Utzinger J., Wu Z., Chen J.X., Chen S.H., Zhang L. 2007. Molecular epidemiology of human *Blastocystis* in a village in Yunnan province, China. *Parasitology International* 56: 281-286.

[38] Ramirez J.D., Sanchez L.V., Bautista D.C., Corredor A.F., Florez A.C., Stensvold C.R. 2014. *Blastocystis* subtypes detected in humans and animals from Colombia. *Infection, Genetics and Evolution* 22: 223-228.

[39] Alfellani M.A., Jacob A.S., Perea N.O., Krecek R.C., Taner-Mulla D., Verweij J.J., Levecke B., Tannich E., Clark C.G., Stensvold C.R. 2013. Diversity and distribution of *Blastocystis* sp. subtypes in non-human primates. *Parasitology* 140: 966-971.

[40] Bart A., Wentink-Bonnema E.M., Gilis H., Verhaar N., Wassenaar C.J., van Vugt M., Goorhuis A., van Gool T. 2013. Diagnosis and subtype analysis of *Blastocystis* sp. in 442 patients in a hospital setting in the Netherlands. *BMC Infectious Diseases* 13: 389. doi:10.1186/1471-2334-13-389.

[41] El Safadi D., Meloni D., Poirier P., Osman M., Cian A., Gaayeb L., Wawrzyniak I., Delbac F., El Alaoui H., Delhaes L. 2013. Molecular epidemiology of *Blastocystis* in Lebanon and correlation between subtype 1 and gastrointestinal symptoms. *American Journal of Tropical Medicine and Hygiene* 88: 1203-1206.

[42] Wong K.H., Ng G., Lin R.T., Yoshikawa H., Taylor M.B., Tan K.S. 2008. Predominance of subtype 3 among *Blastocystis* isolates from a major hospital in Singapore. *Parasitology Research* 102: 663-670.

[43] Jantermtor S., Pinlaor P., Sawadpanich K., Pinlaor S., Sangka A., Wilailuckana C., Wongsena W., Yoshikawa H. 2013. Subtype identification of *Blastocystis* spp. isolated from patients in a major hospital in northeastern Thailand. *Parasitology Research* 112: 1781-1786.

[44] Popruk S., Udonsom R., Koompapong K., Mahittikorn A., Kusolsuk T., Ruangsittichai J., Palasuwan A. 2015. Subtype distribution of *Blastocystis* in Thai-Myanmar Border, Thailand. *Korean Journal of Parasitology* 53: 13-19.

[45] Thathaisong U., Siripattanapipong S., Mungthin M., Pipatsatitpong D., Tan-ariya P., Naaglor T., Leelayoova S. 2013. Identification of *Blastocystis* subtype 1 variants in the home for girls, Bangkok, Thailand. *American Journal of Tropical Medicine and Hygiene* 88: 352-358.

[46] Lee L.I., Chye T.T., Karmacharya B.M., Govind S.K. 2012. *Blastocystis* sp.: waterborne zoonotic organism, a possibility? *Parasites and Vectors* 5: 130. doi: 10.1186/1756-3305-5-130.

[47] Abdulsalam A.M., Ithoi I., Al-Mekhlafi H.M., Ahmed A., Surin J., Mak J.W. 2012. Drinking water is a significant predictor of *Blastocystis* infection among rural Malaysian primary schoolchildren. *Parasitology* 139: 1014-1020.

[48] Leelayoova S., Siripattanapipong S., Thathaisong U., Naaglor T., Taamasri P., Piyaraj P., Mungthin M. 2008. Drinking water: a possible source of *Blastocystis* spp. subtype 1 infection in schoolchildren of a rural community in central Thailand. *American Journal of Tropical Medicine and Hygiene* 79: 401-406.

[49] Leelayoova S., Rangsin R., Taamasri P., Naaglor T., Thathaisong U., Mungthin M. 2004. Evidence of waterborne transmission of *Blastocystis hominis*. *American Journal of Tropical Medicine and Hygiene* 70: 658-662.

[50] Ithoi I., Jali A., Mak J., Wan Sulaiman W.Y., Mahmud R. 2011. Occurrence of *Blastocystis* in water of two rivers from recreational areas in Malaysia. *Journal of Parasitology Research.* doi:10.1155/2011/123916.

Received 11 March 2016
Accepted 10 May 2016