Detection and subtype identification of *Blastocystis* isolates from wastewater samples in the Philippines

Jan Ervin G. Banaticla and Windell L. Rivera

ABSTRACT

To provide further evidence of waterborne transmission of *Blastocystis*, a total of 31 wastewater treatment plants from geographically distinct locations across the Philippines were sampled for influent and effluent sewage samples. *In vitro* cultivation was the method of choice to increase sensitivity of detection. *Blastocystis* cysts were detected in 15% (9/62) of the samples using *in vitro* culture. Moreover, influent and effluent samples were 23% (7/31) and 7% (2/31) positive for the parasite, respectively. The presence of viable cysts in treated samples may be an indication of the inefficiency of the treatment process in preventing *Blastocystis* from entering the environment. Polymerase chain reaction and sequencing of the full-length small subunit ribosomal RNA (SSU rRNA) genes of the nine wastewater isolates were performed. The SSU rRNA gene sequences of the isolates showed very high similarity (98 to 99%) to homologous sequences of *Blastocystis* described previously. The phylogenetic tree constructed showed that the wastewater isolates clustered with each other with good bootstrap support and belonged to two subtypes (ST) – ST1 and ST2. This is the first report of subtyping *Blastocystis* isolates from wastewater samples and gives further emphasis to the remarkable genetic diversity of the parasite.

Key words | *Blastocystis*, Philippines, small subunit ribosomal RNA (SSU rRNA) gene, subtype, wastewater, waterborne transmission

Jan Ervin G. Banaticla
Windell L. Rivera (corresponding author)
Institute of Biology,
College of Science,
University of the Philippines,
Diliman,
Quezon City 1101, Philippines
and
Molecular Protozoology Laboratory,
Natural Sciences Research Institute,
University of the Philippines,
Diliman,
Quezon City 1101, Philippines
Fax: +63 2 920 5471
E-mail: *wlrivera@science.upd.edu.ph*

INTRODUCTION

Blastocystis sp. is one of the most frequently reported protozoan parasites in faecal samples. The parasite thrives in the human gastrointestinal tract and is strictly anaerobic in axenic culture (Tan 2008; Tan *et al.* 2010). Recent data suggest that it has a worldwide distribution with prevalence ranging from 30 to 50% in developing countries, while a lower rate exists in developed countries (Yoshikawa *et al.* 2004*a*; Özyurt *et al.* 2008; Tan 2008; Wong *et al.* 2008; Souppart *et al.* 2010; Tan *et al.* 2010). Owing to its highly zoonotic potential, *Blastocystis* sp. has been isolated in a wide array of hosts including, but not limited to, the following: monkeys, pigs, cattle, birds, amphibians, rodents, reptiles and insects (Noël *et al.* 2005; Rivera 2008; Yoshikawa *et al.* 2009). More recently, three new subtypes (ST) of *Blastocystis* have been reported: ST11, ST12 and ST13 from elephants, giraffes and quokkas, respectively (Parkar *et al.* 2010).

Extensive polymorphism is exhibited by *Blastocystis*, with four major forms observed in culture studies: vacuolar, granular, amoeboid and cyst. The cyst form, which has a small size (2–5 μm) and is mostly ovoid and spherical in shape, was discovered more than a decade ago and is easily confused with faecal debris (Moe *et al.* 1996, 1999). It has been demonstrated that the cyst is the transmissible form of the parasite and that as few as 10 to 100 cysts are sufficient to establish an infection (Yoshikawa *et al.* 2004*b*; Tanizaki *et al.* 2005). Apparently, there is a paucity of studies reported in the literature regarding the analysis of *Blastocystis* isolates from sewage samples. Suresh *et al.* (2005) reported that viable cysts

doi: 10.2166/wh.2010.127

of the organism can survive before and after wastewater treatment. The survival of *Blastocystis* cysts in the environment presents an increased risk for the community because of the extensive reuse of wastewater especially in urban regions worldwide. This practice has long been employed because of its economic and considerable benefits to the general public (US EPA 2001; Salgot *et al.* 2006).

The present study aimed to isolate *Blastocystis* cysts in wastewater samples in the Philippines using both *in vitro* cultivation and molecular approaches. Additionally, the isolates recovered were subjected to phylogenetic analysis to clarify the subtype identification of *Blastocystis* in sewage samples.

MATERIALS AND METHODS

Sample collection and processing

A total of 62 samples (31 influent and 31 effluent) from 31 wastewater treatment plants (WTP) located in the Philippines were analysed in this study. The sampling size for each wastewater sample collected was 500 ml (both influent and effluent) and the sample was stored in sterile plastic bottles. Each of the 31 WTPs was sampled twice from January 2009 to February 2010. In this study, influent and effluent samples were obtained separately. Varied sources from geographically distinct locations were considered in the analysis (Table 1). A wide variety of sampling sites were included such as residential areas and shopping malls, as well as a city jail/police headquarters and a zoological facility.

Sewage samples were chilled and transported immediately to the laboratory. The centrifugation procedure was performed according to the methods specified by Suresh *et al.* (2005) with some modifications. Briefly, the sewage samples were centrifuged using a fixed angle, tabletop centrifuge at 11,180 × *g* for 2 min at room temperature until the volume was reduced to around 50 μl with the pellet retained at the bottom of the tube. Further centrifugation was performed, the supernatant discarded and the pellet resuspended to 100 μl of buffer solution consisting of 137 mM NaCl, 19.6 mM Na_2HPO_4, 1.98 mM KH_2PO_4, and 3.78 mM L-asparagine prior to *in vitro* cultivation.

Table 1 | Location and service area in the Philippines covered by wastewater treatment plants (WTP) in this study

Location	Service area covered by the WTP
Quezon City	Residential, commercial, and university
Quezon City	Residential
Quezon City	Residential
Quezon City	Residential
Quezon City	Residential
Parañaque City	Commercial (shopping mall)
Pasig City	Residential
Pasig City	Residential
Pasig City	Residential
Pasig City	Residential
Pasig City	Residential
Pasig City	Residential
Pasig City	Residential
Pasig City	Residential
Pasig City	Residential
Pasig City	Pet shops
City of Manila	Residential
City of Manila	Zoological facility
Makati City	Commercial
Makati City	Commercial
Makati City	Residential
Mandaluyong City	Hospital
Mandaluyong City	Residential
Pasay City	Commercial (shopping mall)
Pasay City	Commercial
Pasay City	Commercial
Pasay City	Commercial (shopping mall)
Cainta, Rizal	Residential
Taguig City	City jail/police headquarters
Boracay Island, Malay, Aklan	Hotel/Resort
Boracay Island, Malay, Aklan	Hotel/Resort
Total: 31 WTPs	

Isolation and *in vitro* cultivation of *Blastocystis*

For *in vitro* cultivation, 20 μl of each resuspended pellet was subjected to culture using the diphasic medium consisting of 1.5% agar overlaid with buffer solution as described above

and supplemented with 10% heat-inactivated horse serum (Gibco^{TM}, Invitrogen Corporation, Auckland, New Zealand) and antibiotics (Rivera 2008). Samples observed under light microscopy exhibiting the common forms of *Blastocystis* (e.g. vacuolar, granular) were considered positive and incubated at 37°C and subcultured every 3 or 4 days.

Genomic DNA extraction, PCR and DNA sequencing

Genomic DNA extraction was performed using phenol/chloroform/isoamyl alcohol (Rivera *et al.* 1996). PCR of the SSU rRNA gene was performed using GoTaq® Green Master Mix (Promega, Madison, WI) and oligonucleotide primers, SR1F and SR1R (Yoshikawa *et al.* 2000). Aliquots (5 μl) of the PCR amplicons were run in 1.5% agarose gels, stained with 0.5 μg ml^{-1} ethidium bromide and viewed under UV transilluminator. Samples were considered positive for *Blastocystis* if a ~1.7 kb DNA fragment was visible. Positive PCR amplicons were subsequently sent to Macrogen, Inc. (Seoul, South Korea) for purification and sequencing. The complete SSU rRNA gene was sequenced using primers, SR1F, F70, ABBH1, B6, B71 and SR1R. The internal primers F70, ABBH1, B6 and B71 were developed by Abe (2004) to prime conserved regions of SSU rDNA sequences obtained from *B. hominis* strains, Nand II (GenBank No. U51151) and NIH-1295-1 (GenBank No. U51152) (Silberman *et al.* 1996).

Subtype identification, sequence alignment and phylogenetic analysis

The subtypes of the Philippine wastewater isolates were identified by searching through an online database of nucleotide sequences using the Basic Local Alignment Search Tool (BLAST) in GenBank of the US National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST, accessed 19 November 2010). The search yielded results with close similarity against known *Blastocystis* subtypes. Multiple sequences were aligned using the ClustalW program of BioEdit v7.0 (Hall 1999) and by ocular inspection to remove gaps and ambiguous sequences. A total of 21 reference isolates representing seven different subtypes of *Blastocystis* were used in this study in addition to the 18S rRNA gene sequence of *Proteromonas lacertae* (Table 2). Phylogenetic trees were constructed and rooted to the sequence of *P. lacertae* owing to its close relationship with *Blastocystis* (Silberman *et al.* 1996; Noël *et al.* 2005). The trees were constructed using neighbour-joining (NJ) and maximum likelihood (ML) which were all based on the TrN (Tamura & Nei 1993) + Γ (gamma distribution of rates with four rate categories) model as well as the non-model-based maximum parsimony (MP). Bootstrap resampling was also carried out with 1,000 replicates for NJ and MP and 100 replicates for ML. PAUP* version 4.0b10 was used for NJ and MP (Swofford 2000) while sequences were uploaded into the website PHYML (http://www.atgc-montpellier.fr/phyml, accessed 19 November 2010) for the ML analysis (Guindon *et al.* 2005). Clusters in the phylogenetic trees were only considered valid if their bootstrap support were >50% for NJ, ML and MP. Trees were viewed using TreeView v. 1.6.6 (Page 1996).

Nucleotide sequence accession numbers

Sequences determined in the present study were deposited in GenBank as accessions GU992411 to GU992419 (Table 3). The reference sequences used are listed in Table 2.

RESULTS AND DISCUSSION

In this study, *in vitro* culture method was performed to detect *Blastocystis* in wastewater samples. Vacuolar and granular forms of *Blastocystis* were observed under light microscopy. For influent and effluent wastewater samples, 23% (7/31) and 7% (2/31) were positive for the parasite, respectively. Therefore, the rate of detection for this study was 15% (9/62) using *in vitro* cultivation. The seven isolates recovered from influent (untreated) samples were from shopping malls (In1 and In8) in the cities of Parañaque and Pasay, respectively, a residential area in Quezon City (In9), a hotel/resort in Boracay Island, Malay, Aklan (In10 and In11), a zoological facility from the City of Manila (In6), and a city jail/police headquarters in Taguig City (In7) (Table 3). In contrast, the two isolates acquired from effluent (treated) samples (Ef1 and Ef3) were both from residential areas in Cainta, Rizal, and Pasig City, respectively (Table 3).

Culture-positive samples were subjected to PCR to augment the detection method employed. The isolates were 100% (9/9) positive for PCR using the specific primers,

Table 2 | GenBank reference sequences of *Blastocystis* used in this study

GenBank accession number	Reference isolate	Host	Subtype
AB070989	*Blastocystis* sp. HJ96A-29	Human	1
AB107967	*Blastocystis* sp. MJ99-424	Monkey	1
U51151	*Blastocystis hominis* strain Nand II	Human	1
AB070987	*Blastocystis hominis* HJ96-1	Human	2
AB070997	*Blastocystis* sp. JM92-2	Monkey	2
AB107969	*Blastocystis* sp. MJ99-116	Monkey	2
AB070988	*Blastocystis hominis* HJ96-A26	Human	3
AB107963	*Blastocystis* sp. PJ99-162	Pig	3
AB107965	*Blastocystis* sp. CJ99-363	Cattle	3
AB071000	*Blastocystis* sp. RN94-9	Rat	4
U51152	*Blastocystis* sp. NIH:1295:1	Guinea pig	4
AY135408	*Blastocystis* sp. clone 2	Rat	4
AB070999	*Blastocystis* sp. SY94-7	Pig	5
AB107964	*Blastocystis* sp. PJ99-188	Pig	5
AB070998	*Blastocystis* sp. SY94-3	Pig	5
AB107972	*Blastocystis* sp. BJ99-310	Partridge	6
AB070995	*Blastocystis* sp. QQ93-3	Quail	6
AB070990	*Blastocystis hominis* strain HJ96AS-1	Human	6
AY135412	*Blastocystis* sp.	Duck	7
AB070991	*Blastocystis hominis* HJ97-2	Human	7
AF408427	*Blastocystis hominis* strain B	Human	7
U37108	*Proteromonas lacertae*	Unknown	n/a

n/a – not applicable

SR1F and SR1R. After purification and sequencing of the PCR products, the subtypes of the isolates were identified using BLAST search by determining the closest similarity against known *Blastocystis* subtypes. The length of SSU rRNA gene sequences obtained ranged from 1,693 bp (In8) to 1,764 bp (In1). Each of the new SSU rRNA gene sequences of the wastewater isolates showed very high similarity (from 98 to 99%) to homologous sequences of other *Blastocystis* isolates reported so far (Table 3). In this investigation, two subtypes in the highly variable regions of SSU rRNA gene were present after alignment of sequences. Subtype 1 (ST1) consisted of seven isolates, six from untreated sewage water (In1, In7, In8, In9, In10 and In11), and one from treated sewage water (Ef3). Subtype 2 (ST2) consisted of two isolates, one from untreated sewage water (In6) and one from treated sewage water (Ef1).

Molecular phylogenetic analysis was performed using the SSU rDNA sequences of the nine wastewater isolates along with 21 reference sequences of *Blastocystis* from GenBank. The use of *P. lacertae* as an outgroup showed that the two subtypes (ST1 and ST2) are separated to form sister clades (Figure 1). The data set used for this tree was based on 1,471 unambiguously aligned positions. The wastewater isolates identified as belonging to ST1 and ST2 formed sister clades with each other with good bootstrap support values of 100/100 (MP and ML, respectively) for ST1 and 100/100/99 (NJ, MP and ML, respectively) for ST2. The rooted neighbour-joining tree of *Blastocystis* after sequence alignment identified seven clades of subtypes 1 to 7 with strong bootstrap support for each clade.

Blastocystis was found to be present in 15% (9/62) of the Philippine wastewater samples using *in vitro* cultivation. In

Table 3 | SSU rRNA gene sequence similarities between *Blastocystis* isolates used in this study and GenBank reference sequences

Isolate	ST	GenBank accession number	Type of wastewater sample	Location	Service area of the WTP	Similar GenBank reference sequence	Sequence similarity (%)
In1	1	GU992411	Influent	Parañaque City, Metro Manila	Shopping mall	*Blastocystis* sp. MJ99-424 (AB107967)	99
In6	2	GU992412	Influent	City of Manila	Zoological facility	*Blastocystis hominis* isolate 418 (AY956324)	98
In7	1	GU992413	Influent	Taguig City, Metro Manila	City jail/Police HQ	*Blastocystis* sp. MJ99-424 (AB107967)	99
In8	1	GU992414	Influent	Pasay City, Metro Manila	Shopping mall	*Blastocystis* sp. MJ99-424 (AB107967)	99
In9	1	GU992415	Influent	Quezon City, Metro Manila	Residential	*Blastocystis* sp. M2 (EU445488)	99
In10	1	GU992416	Influent	Boracay Island, Malay, Aklan	Hotel/Resort	*Blastocystis* sp. MJ99-424 (AB107967)	99
In11	1	GU992417	Influent	Boracay Island, Malay, Aklan	Hotel/Resort	*Blastocystis* sp. MJ99-424 (AB107967)	99
Ef1	2	GU992418	Effluent	Cainta, Rizal	Residential	*Blastocystis hominis* HJ96-1 (AB070987)	99
Ef3	1	GU992419	Effluent	Pasig City, Metro Manila	Residential	*Blastocystis* sp. M2 (EU445488)	98

comparison, Scottish and Malaysian sewage samples were 18% (13/73) and 68% (34/50) positive for *Blastocystis*, respectively (Suresh *et al.* 2005). For effluents or treated samples in the Philippines, 7% (2/31) were positive for *Blastocystis* while effluents in Scotland and Malaysia were 9% (4/42) and 60% (15/25) positive for the parasite, respectively. Taking into account the distinct geographical locations of the wastewater treatment plants sampled in the present and previous analyses, it is safe to assume that the wastewater treatment processes currently employed may be ineffective in removing and preventing *Blastocystis* cysts from being released in the environment. This presents a parasitological risk for public health because wastewater reuse is a common method in urban regions around the world (US EPA 2001; Salgot *et al.* 2006). Treated wastewater has a number of uses such as landscape irrigation, vehicle washing, toilet flushing, fire protection, commercial air conditioners and other similar purposes (US EPA 1992). This extensive reuse of wastewater reveals increased exposure of humans to a number of parasites. The removal efficiencies of the treatment process for *Blastocystis* were not determined in this study since the samples were collected separately. In contrast, the removal efficiencies for *Cryptosporidium* spp. and *Giardia duodenalis* are well-established (Burkhari *et al.* 1997; Cacciò *et al.* 2003; Robertson *et al.* 2006; Castro-Hermida *et al.* 2008). These parasites are transmitted through contaminated food and water during the transmissible stages in their life-cycle: oocyst for *Cryptosporidium* and cyst for *G. duodenalis* (Fayer *et al.* 2000; Slifko *et al.* 2000; Thompson 2000). Castro-Hermida *et al.* 2008 reported a 100% detection rate for *Cryptosporidium* spp. and *G. duodenalis* in both influents and effluents for all treatment plants included in their case study. It should be noted, however, that from a statistical standpoint, the sample size (500 ml) considered in this analysis is not large enough to establish the inefficiency of the treatment process. This warrants extensive collection and more samples from various wastewater treatment plants.

In vitro cultivation was employed to detect *Blastocystis* in Philippine wastewater samples. Several studies have shown that *in vitro* culture proved to be more sensitive in detecting the organism compared with direct microscopic observation (Zaman & Khan 1994; Suresh *et al.* 1997; Leelayoova *et al.* 2002; Suresh & Smith 2004; Termmathurapoj *et al.* 2004; Stensvold *et al.* 2007). The culture method increases the percentage positivity rate for viable organisms because of the increase at an exponential rate in a medium and allows

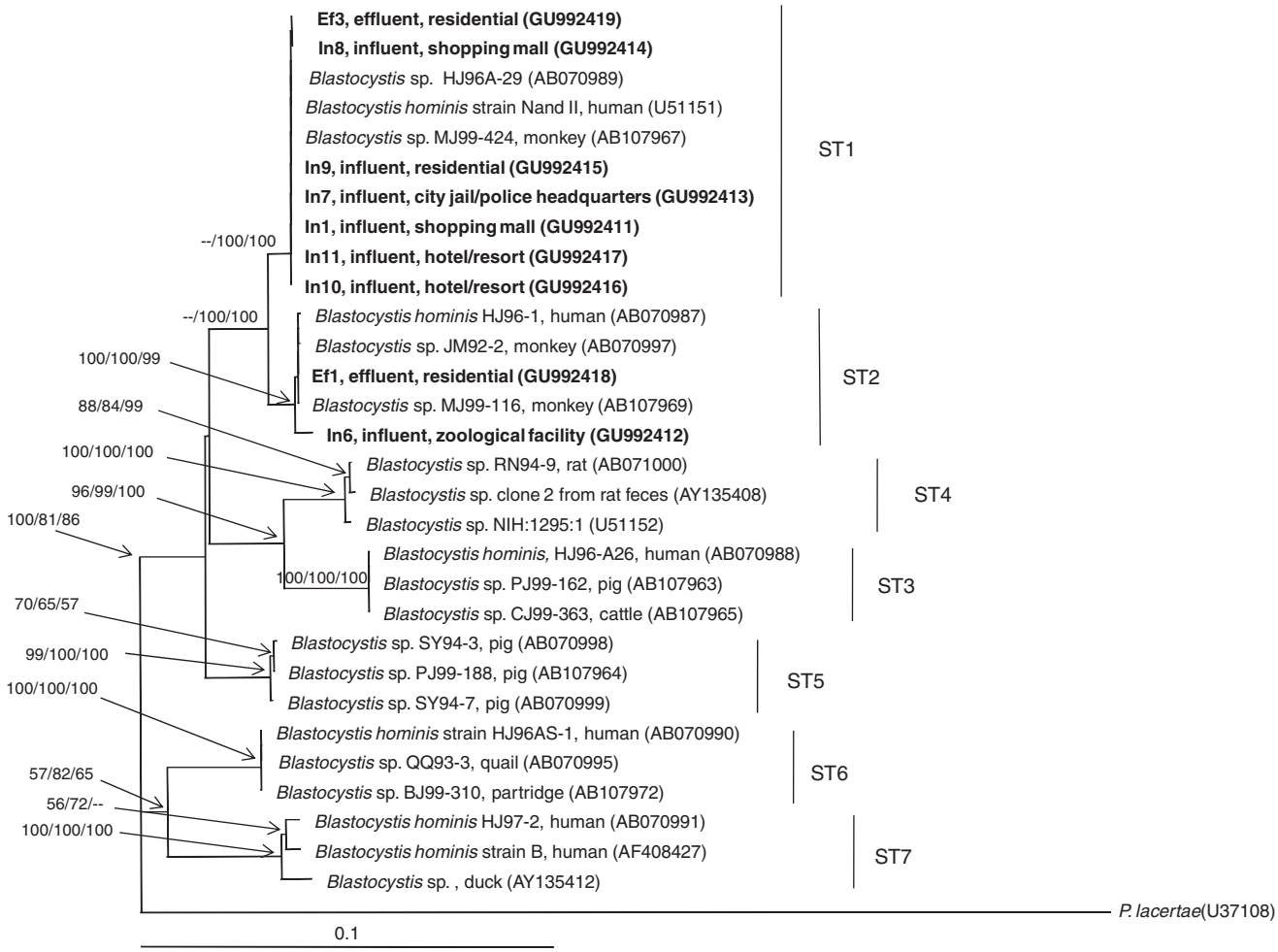

Figure 1 | Molecular phylogeny of *Blastocystis* isolates inferred from 1,471 unambiguously aligned basepairs of the SSU rRNA gene sequences and using neighbour-joining analysis. *Proteromonas lacertae* served as the outgroup. Bootstrap support values on the nodes are from neighbour-joining, maximum parsimony and maximum likelihood, respectively. Values of $<50\%$ are not shown. *Blastocystis* isolates from wastewater samples in the Philippines are indicated in bold. Scale bar, 0.1 substitutions per base pair.

low numbers of *Blastocystis* to be cultured for use in either genotypic or phenotypic assays (Suresh & Smith 2004). The results of this study are consistent with those of Suresh et al. in 2005 who also utilized *in vitro* culture in detecting *Blastocystis* in sewage samples. Previous studies have chosen Jones' medium to identify the organism in stool samples from patients (Leelayoova *et al.* 2002; Parkar *et al.* 2007). In addition, wastewater isolates of *Blastocystis* exhibited good growth in Jones' medium as shown by Suresh *et al.* (2005). However, there was a report of unsuccessful cultivation of *Blastocystis* isolates from Australian marsupials in Jones' medium (Parkar *et al.* 2007), which may suggest a limitation of the medium in supporting the growth of other isolates from different hosts. An alternative would be the diphasic agar slant medium which proved to be useful in culturing isolates from different hosts (e.g. cattle, chickens, pigs) (Abe *et al.* 2003*a*, *b*, *c*; Rivera 2008). A recent report showed that Philippine *Blastocystis* isolates from humans, monkeys, pigs and chickens grow abundantly using a diphasic medium supplemented with 10% horse serum and antibiotics (Rivera 2008). The same diphasic medium was also utilized in this study and was confirmed to be useful in detecting and maintaining environmental isolates.

Despite the advantage of using *in vitro* propagation, due to its increased sensitivity in detecting *Blastocystis*, isolates grown in culture may inaccurately depict the parasite population sampled. In the case of mixed infections wherein a host carries more than one subtype, *in vitro* methods may favour

the preferential amplification of one subtype over the other (Parkar *et al.* 2007; Yan *et al.* 2007). For instance, the avian subtype, ST7 has a longer doubling time, about 50 h, when incubated at 37°C (Ho *et al.* 1993). Slow-growing subtypes are therefore at risk of being under-represented during propagation in stool samples. Wastewater samples come from varied sources and may possibly harbour a plethora of subtypes. Although samples in this analysis came from varied sources, only two subtypes were detected, ST1 and ST2 (Table 3). To determine the true distribution of subtypes, it is prudent to directly genotype *Blastocystis* DNA from stool samples as suggested by recent literature (Tan 2008; Tan *et al.* 2010). A previous study comparing detection methods for the diagnosis of *Blastocystis* infections revealed that out of 107 samples, 42% and 19% were positive using PCR and *in vitro* culture, respectively (Parkar *et al.* 2007). This suggests the possibility of directly detecting *Blastocystis* from faeces with greater sensitivity. Moreover, the same study by Parkar *et al.* 2007 demonstrated that ST5 overgrew ST1 *in vitro*. In the case of sewage samples, no protocol currently exists that directly genotypes *Blastocystis* DNA. It may be worthwhile to explore the use of immunomagnetic separation technology as this is already being utilized for the direct detection of *Cryptosporidium* spp. and *G. duodenalis* (oo)cysts in sewage samples (US EPA 2001; Castro-Hermida *et al.* 2008). Nevertheless, this is the first report of subtyping *Blastocystis* isolates from sewage samples and provides evidence of the possibility of waterborne transmission through viable cysts in wastewater.

The present analysis identified ST1 in 78% (7/9) of the culture-positive samples as confirmed by PCR and sequencing while ST2 was present in 22% (2/9) of the samples (Table 3). Multiple epidemiological surveys conducted in numerous countries implicate *Blastocystis* sp. ST3 as the most common and 'true' human subtype (Özyurt *et al.* 2008; Tan 2008; Wong *et al.* 2008; Souppart *et al.* 2010; Tan *et al.* 2010). However, a report showed that ST1 was common in Thailand (Leelayoova *et al.* 2008) while another case study also found that ST1 has a relatively high proportion (37.1%) in humans in Jiangxi, China (Yan *et al.* 2006). In the Philippines, the most dominant genotype was ST3 among human isolates while the animal isolates showed varied subtypes (ST1, ST2, ST3 and ST6) (Rivera 2008). However, Rivera & Tan (2005) also reported that 10 out of 12 human isolates in the Philippines were ST1 based on RFLP (restriction fragment length polymorphism) analysis of the SSU rRNA gene. This evidence of waterborne transmission is attributed to the cyst form of the parasite which is the transmissible stage. Hosts continually excrete cysts through contamination of food, environment and water (Singh *et al.* 1995; Taamasri *et al.* 2000; Leelayoova *et al.* 2004; Suresh *et al.* 2005; Li *et al.* 2007). Reports of waterborne transmission of the parasite due to contamination of drinking water with infective cysts have been well-documented in Thailand (Leelayoova *et al.* 2004, 2008). In 1996, Zaki and colleagues demonstrated that *Blastocystis* cysts can withstand chlorination at standard concentrations (Zaki *et al.* 1996). The present analysis revealed that both ST1 (Ef3) and ST2 (Ef1) (Table 3) were present in treated sewage samples which had undergone chlorination and may indicate that these subtypes can withstand sewage treatment procedures and remain viable in the environment. In addition, the results also show the sporadic nature of *Blastocystis* in environmental samples wherein several sites yielded positive results in effluents while negative for influents (Table 3).

This study provides evidence that *Blastocystis* cysts can survive in varied settings in the environment. Strict guidelines and regulations stipulate that wastewater treatment plants be established especially in urban regions worldwide as a vital step in protecting the environment. Wastewater professionals have developed methods for wastewater reuse because of its significant economic and environmental benefits to the community (US EPA 2001; Salgot *et al.* 2006). Similarly, urban regions in the Philippines have wastewater treatment plants covering different service areas (Table 1). Five ST1 isolates from different sources are determined to be 99% similar to a monkey reference isolate, MJ99-424, stated in another study (GenBank accession no. AB107967; Abe 2004). These isolates were recovered from influents of wastewater treatment plants situated in the following places: shopping malls in Metro Manila (In1 and In 8), a hotel/resort in Boracay Island, Malay, Aklan (In10 and In11), and a city jail/police headquarters in Taguig City (In7). Two ST1 isolates, one from influent (In9) and one from effluent (Ef3), are 99 and 98% homologous to another monkey reference isolate, M2, respectively (GenBank accession no. EU445488; Rivera 2008). In contrast, one ST2 isolate (In6) has a 98% sequence similarity to a human reference isolate, 418 (GenBank acces-

sion no. AY956324). This isolate was recovered from an influent sample of a treatment plant in a zoological facility in the city of Manila. The other ST2 isolate (Ef1) is 99% homologous to a human reference isolate, HJ96-1, described in a previous report (GenBank accession no. AB070987; Arisue *et al*. 2003). Our study, however, does not conclusively imply that wastewater isolates which are ST1 and ST2 are of monkey and human origins, respectively. This is due to the cross-infectivity of ST1 isolates among humans, monkeys, pigs and chickens while ST2 isolates have been found in humans, monkeys and pigs (Tan 2008). Furthermore, the cysts recovered from wastewater samples might be mixed with different origins as stated above. Nevertheless, the presence of different subtypes in treatment plants covering a wide range of service areas (Table 3) confirms the remarkable ability of *Blastocystis* cysts to survive in diverse conditions in the environment before and after wastewater treatment.

The present investigation confirms the genetic diversity of *Blastocystis* isolates from environmental samples based on the phylogenetic tree generated (Figure 1). The major clades identified in this analysis were in agreement with previous phylogenetic studies which utilized the SSU rDNA sequence (Arisue *et al*. 2003; Abe 2004; Noël *et al*. 2005; Rivera 2008). The present phylogeny showed *Blastocystis* sp. ST1 and ST2 isolates from sewage samples with good bootstrap support for each clade. The subtypes detected in this study (ST1 and ST2) have been implicated for zoonotic transmission and cross-transmissibility among human and animal hosts (Noël *et al*. 2005; Rivera 2008). This is the first report of molecular phylogenetic analysis of *Blastocystis* from wastewater samples. The tree based on the SSU rDNA sequences clarified the genetic diversity and subtypes of the isolates.

The predominance of ST1 (78%) among *Blastocystis* isolates from sewage samples raises public health issues, especially if the parasitological risk of wastewater reuse is taken into account. Recently, Hussein *et al*. (2008) demonstrated that *Blastocystis* sp. ST1 recovered from both asymptomatic and symptomatic patients in Egypt can induce up to 25% mortality in rats and led to the conclusion that the subtype is pathogenic. Another case study demonstrated that ST1 was predominant in a group of symptomatic patients with *Blastocystis* infections (Yan *et al*. 2006). More recently, ST1 was found to be dominant among patients with irritable bowel syndrome-diarrhoea in Pakistan (Yakoob *et al*. 2010). Although the Philippines lack strong epidemiological data on *Blastocystis*, nonetheless, there is a need to develop better strategies in eliminating *Blastocystis* and other protozoan pathogens in the environment. This should be tackled by stakeholders and eventually incorporated into a relevant and responsive public health policy.

CONCLUSION

The present analysis showed that *Blastocystis* isolates can survive in wastewater samples before and after treatment. These findings suggest that viable cysts have the capability to withstand several treatment processes currently employed. ST1 was predominant in the majority of the samples as confirmed by PCR and sequencing. A comprehensive molecular epidemiology of *Blastocystis* isolates in the Philippines is needed as baseline data for further research. Lastly, the survival of different subtypes of *Blastocystis* in sewage samples should raise public health concerns given that wastewater reuse is extensive in urban regions worldwide.

ACKNOWLEDGEMENTS

We thank Davin Edric V. Adao, Marjo V. Villareal and Carla Bianca L. Victorio for technical assistance. This work was supported by grants from the Natural Sciences Research Institute, University of the Philippines Diliman and the University of the Philippines System (to W.L.R.) and the Philippine Council for Advanced Science and Technology Research and Development of the Department of Science and Technology (to J.E.G.B.).

REFERENCES

Abe, N. 2004 Molecular and phylogenetic analysis of *Blastocystis* isolates from various hosts. *Vet. Parasitol.* **120**, 235–242.

Abe, N., Wu, Z. & Yoshikawa, H. 2003*a* Molecular characterization of *Blastocystis* isolates from birds by PCR with diagnostic primers and restriction fragment length polymorphism analysis of the small subunit ribosomal RNA gene. *Parasitol. Res.* **89**, 393–396.

Abe, N., Wu, Z. & Yoshikawa, H. 2003*b* Molecular characterization of *Blastocystis* isolates from primates. *Vet. Parasitol.* **113**, 321–325.

Abe, N., Wu, Z. & Yoshikawa, H. 2003*c* Zoonotic genotypes of *Blastocystis hominis* detected in cattle and pigs by PCR with diagnostic primers and restriction fragment length polymorphism analysis of the small subunit ribosomal RNA gene. *Parasitol. Res.* **90**, 124–128.

Arisue, N., Hashimoto, T. & Yoshikawa, H. 2003 Sequence heterogeneity of the small subunit ribosomal RNA genes among *Blastocystis* isolates. *Parasitology* **126**, 1–9.

Burkhari, Z., Smith, H. V., Sykes, N., Humphreys, S. W., Paton, C. A., Girdwood, R. W. & Fricker, C. R. 1997 Occurrence of *Cryptosporidium* spp. oocysts and *Giardia* cysts in sewage influents and effluents from treatment plants in England. *Water Sci. Technol.* **35**, 385–390.

Cacciò, S. M., De Giacomo, M., Aulicino, F. A. & Pozio, E. 2003 *Giardia* cysts in wastewater treatment plants in Italy. *Appl. Environ. Microbiol.* **69**, 3393–3398.

Castro-Hermida, J. A., Garciá-Presedo, I., Almeida, A., González-Warleta, M., Da Costa, J. M. & Mezo, M. 2008 Contribution of treated wastewater to the contamination or recreational river areas with *Cryptosporidium* spp. and *Giardia duodenalis*. *Water Res.* **42**, 3528–3538.

Fayer, R., Morgan, U. & Upton, S. J. 2000 Epidemiology of *Cryptosporidium*: transmission, detection and identification. *Int. J. Parasitol.* **30**, 1305–1322.

Guindon, S., Letheic, F., Duroux, P. & Gascuel, O. 2005 PHYML online: A web server for fast maximum likelihood-based phylogenetic inference. *Nucleic Acids Res.* **33**, W557–W559 (web server issue).

Hall, T. A. 1999 BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* **41**, 95–98.

Ho, L. C., Singh, M., Suresh, G., Ng, G. C. & Yap, E. H. 1993 Axenic culture of *Blastocystis hominis* in Iscove's modified Dulbecco's medium. *Parasitol. Res.* **79**, 614–616.

Hussein, E. M., Hussein, A. M., Eida, M. M. & Atwa, M. M. 2008 Pathophysiological variability of different genotypes of human *Blastocystis hominis* Egyptian isolates in experimentally infected rats. *Parasitol. Res.* **102**, 853–860.

Leelayoova, S., Rangsin, R., Taamasri, P., Naaglor, T., Thathaisong, U. & Mungthin, M. 2004 Evidence of waterborne transmission of *Blastocystis hominis*. *Am. J. Trop. Med. Hyg.* **70**, 658–662.

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. *Am. J. Trop. Med. Hyg.* **79**, 401–406.

Leelayoova, S., Taamasri, P., Rangsin, R., Naaglor, T., Thathaisong, U. & Mungthin, M. 2002 In vitro cultivation: a sensitive method for detecting *Blastocystis hominis*. *Ann. Trop. Med. Parasitol.* **96**, 803–807.

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. *Parasitol. Int.* **56**, 281–286.

Moe, K. T., Singh, M., Howe, J., Ho, L. C., Tan, S. W., Chen, X. Q. & Yap, E. H. 1999 Development of *Blastocystis hominis* cysts into vacuolar forms *in vitro*. *Parasitol. Res.* **85**, 103–108.

Moe, K. T., Singh, M., Howe, J., Ho, L. C., Tan, S. W., Ng, G. C., Chen, X. Q. & Yap, E. H. 1996 Observations on the ultrastructure and viability of the cystic stage of *Blastocystis hominis* from human feces. *Parasitol. Res.* **82**, 439–444.

Noël, C., Dufernez, F., Gerbod, D., Edgcomb, V. P., Delgado-Viscogliosi, P., Ho, L. C., Singh, M., Wintjens, R., Sogin, M. L., Capron, M., Pierce, R., Zenner, L. & Viscogliosi, E. 2005 Molecular phylogenies of *Blastocystis* isolates from different hosts: implications for genetic diversity, identification of species, and zoonosis. *J. Clin. Microbiol.* **43**, 348–355.

Özyurt, M., Kurt, O., Mølbak, K., Nielsen, H. V., Haznedaroglu, T. & Stensvold, C. R. 2008 Molecular epidemiology of *Blastocystis* infections in Turkey. *Parasitol. Int.* **57**, 300–306.

Page, R. D. 1996 TREEVIEW: an application to display phylogenetic trees on personal computers. *CABIOS* **12**, 357–358.

Parkar, U., Traub, R. J., Kumar, S., Mungthin, M., Vitali, S., Leelayoova, S., Morris, K. & Thompson, R. C. 2007 Direct characterization of *Blastocystis* from faeces by PCR and evidence of zoonotic potential. *Parasitology* **134**, 359–367.

Parkar, U., Traub, R. J., Vitali, S., Elliot, A., Levecke, B., Robertson, I., Geurden, T., Steele, J., Drake, B. & Thompson, R. C. 2010 Molecular characterization of *Blastocystis* isolates from zoo animals and their animal-keepers. *Vet. Parasitol.* **169**, 8–17.

Rivera, W. L. 2008 Phylogenetic analysis of *Blastocystis* isolates from animal and human hosts in the Philippines. *Vet. Parasitol.* **156**, 178–182.

Rivera, W. L., Tachibana, H., Silva-Tahat, M. R., Uemura, H. & Kanbara, H. 1996 Differentiation of *Entamoeba histolytica* and *E. dispar* DNA from cysts present in stool specimens by polymerase chain reaction: its field application in the Philippines. *Parasitol. Res.* **82**, 585–589.

Rivera, W. L. & Tan, M. A. 2005 Molecular characterization of *Blastocystis* isolates in the Philippines by riboprinting. *Parasitol. Res.* **96**, 253–257.

Robertson, L. J., Hermansen, L. & Gjerde, B. K. 2006 Occurrence of *Cryptosporidium* oocysts and *Giardia* cysts in sewage in Norway. *Appl. Environ. Microbiol.* **72**, 5297–5303.

Salgot, M., Huertas, E., Weber, S., Dott, W. & Hollender, J. 2006 Wastewater reuse and risk: definition of key objectives. *Desalination* **187**, 29–40.

Silberman, J. D., Sogin, M. L., Leipe, D. D. & Clark, C. G. 1996 Human parasite finds taxonomic home. *Nature* **380**, 398.

Singh, M., Suresh, K., Ho, L. C., Ng, G. C. & Yap, E. H. 1995 Elucidation of the life cycle of the intestinal protozoan *Blastocystis hominis*. *Parasitol. Res.* **81**, 446–450.

Slifko, T. R., Smith, H. V. & Rose, J. B. 2000 Emerging parasite zoonoses associated with water and food. *Int. J. Parasitol.* **30**, 1379–1393.

Souppart, L., Moussa, H., Cian, A., Sanciu, G., Poirier, P., El Alaoui, H., Delbac, F., Boorom, K., Delhaes, L., Dei-Cas, E. & Viscogliosi, E. 2010 Subtype analysis of *Blastocystis* isolates from symptomatic patients in Egypt. *Parasitol. Res.* **106**, 505–511.

Stensvold, C. R., Suresh, G. K., Tan, K. S., Thompson, R. C., Traub, R. J., Viscogliosi, E., Yoshikawa, H. & Clark, C. G. 2007 Terminology for *Blastocystis* subtypes: A consensus. *Trends Parasitol.* **23**, 93–96.

Suresh, K., Mak, J. W., Chuong, L. S., Ragunathan, T. & Init, I. 1997 Sac-like pouches in *Blastocystis* from the house lizard *Cosymbotus platyurus*. *Parasitol. Res.* **83**, 523–525.

Suresh, K. & Smith, H. 2004 Comparison of methods for detecting *Blastocystis hominis*. *Eur. J. Clin. Microbiol. Infect. Dis.* **23**, 509–511.

Suresh, K., Smith, H. V. & Tan, T. C. 2005 Viable *Blastocystis* cysts in Scottish and Malaysian sewage samples. *Appl. Environ. Microbiol.* **71**, 5619–5620.

Swofford, D.L. 2000 *PAUP*. In: Phylogenetic Analysis using Parsimony (*and other Methods). V. 4.0b10,* Sinauer Associates, Sunderland, Massachusetts.

Taamasri, P., Mungthin, M., Rangsin, R., Tongupprakarn, B., Areekul, W. & Leelayoova, S. 2000 Transmission of intestinal blastocystosis related to the quality of drinking water. *Southeast Asian J. Trop. Med. Public Health* **31**, 112–117.

Tamura, K. & Nei, M. 1993 Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.* **10**, 512–526.

Tan, K. S. W. 2008 New insights on classification, identification, and clinical relevance of *Blastocystis* spp. *Clin. Microbiol. Rev.* **21**, 639–665.

Tan, K. S. W., Mirza, H., Teo, J. D. W., Wu, B. & MacAry, P. A. 2010 Current views on the clinical relevance of *Blastocystis* spp. *Curr. Infect. Dis. Rep.* **12**, 28–35.

Tanizaki, A., Yoshikawa, H., Iwatani, S. & Kimata, I. 2005 Infectivity of *Blastocystis* isolates from chickens, quails and geese in chickens. *Parasitol. Res.* **96**, 57–61.

Termmathurapoj, S., Leelayoova, S., Aimpun, P., Thathaisong, U., Nimmanon, T., Taamasri, P. & Mungthin, M. 2004 The usefulness of short-term in vitro cultivation for the detection and molecular study of *Blastocystis hominis* in stool specimens. *Parasitol. Res.* **93**, 445–447.

Thompson, R. C. 2000 Giardiasis as a re-emerging infectious disease and its zoonotic potential. *Int. J. Parasitol.* **30**, 1259–1267.

US Environmental Protection Agency 1992 *Guidelines for Water Reuse*, (EPA)/625/R-92/004. EPA, Washington, DC.

US Environmental Protection Agency 2001 Method 1623: *Cryptosporidium* and *Giardia* in water by filtration/IMS/FA. EPA 821-R-01-025. Office of Ground Water and Drinking Water Technical Support Center, US Environmental Protection Agency, Washington, DC.

Wong, K. H., Ng, G. C., 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. *Parasitol. Res.* **102**, 663–670.

Yakoob, J., Jafri, W., Beg, M. A., Abbas, Z., Naz, S., Islam, M. & Khan, R. 2010 Irritable bowel syndrome: is it associated with genotypes of *Blastocystis hominis*?. *Parasitol. Res.* **106**, 1033–1038.

Yan, Y., Su, S., Lai, R., Liao, H., Ye, J., Li, X., Luo, X. & Chen, G. 2006 Genetic variability of *Blastocystis hominis* isolates in China. *Parasitol. Res.* **99**, 597–601.

Yan, Y., Su, S., Ye, J., Lai, X., Lai, R., Liao, H., Chen, G., Zhang, R., Hou, Z. & Luo, X. 2007 *Blastocystis* sp. subtype 5: a possibly zoonotic genotype. *Parasitol. Res.* **101**, 1527–1532.

Yoshikawa, H., Abe, N., Iwasawa, M., Kitano, S., Nagano, I., Wu, Z. & Takahashi, Y. 2000 Genomic analysis of *Blastocystis hominis* strains isolated from two long-term health care facilities. *J. Clin. Microbiol.* **38**, 1324–1330.

Yoshikawa, H., Wu, Z., Kimata, I., Iseki, M., Ali, I. K., Hossain, M. B., Zaman, V., Haque, R. & Takahashi, Y. 2004a Polymerase chain reaction-based genotype classification among human *Blastocystis hominis* populations isolated from different countries. *Parasitol. Res.* **92**, 22–29.

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. *Vet. Parasitol.* **160**, 295–300.

Yoshikawa, H., Yoshida, K., Nakajima, A., Yamanari, K., Iwatani, S. & Kimata, I. 2004b Fecal-oral transmission of the cyst form of *Blastocystis hominis* in rats. *Parasitol. Res.* **94**, 391–396.

Zaki, M., Zaman, V. & Sheikh, N. A. 1996 Resistance of *Blastocystis hominis* cysts to chlorine. *J. Pak. Med. Assoc.* **46**, 178–179.

Zaman, V. & Khan, K. Z. 1994 A concentration technique of obtaining viable cysts of *Blastocystis hominis* from faeces. *J. Pak. Med. Assoc.* **44**, 220–221.

First received 21 July 2010; accepted in revised form 13 September 2010. Available online 15 December 2010