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ORIGINAL PAPER

Predominance of subtype 3 among *Blastocystis* isolates from a major hospital in Singapore

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Abstract *Blastocystis* is an enteric protozoan parasite commonly found in humans and animals. Phylogenetic and genotypic analyses have shown that *Blastocystis* exhibits extreme genetic diversity, and humans are host to a number of zoonotic isolates. In the present study, the prevalence of *Blastocystis* in 276 stool samples from a hospital in Singapore was examined, and for the first time, riboprinting using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to determine the genetic diversity of the *Blastocystis* isolated from the Singapore population. The prevalence rate was determined to be 3.3% (9/276), and *Blastocystis* displaying two main ribotypes were isolated. As a comparison, we performed PCR-RFLP using two different published methodologies, and both methods allowed the isolates to be divided into two distinct groups based on their riboprint patterns. According to a recently proposed classification scheme, 78% (7/9) of the isolates were of subtype 3, while 22% (2/9) were subtype 1. The predominance of subtype 3 in an urbanized city state such as Singapore is in agreement with the idea that subtype 3 is a genotype of human origin.

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Introduction

Blastocystis is one of the most common parasites of humans. Epidemiological surveys have shown that prevalence of up to 10% was recorded in developed countries, rising to 50–60% in developing countries (Stenzel et al. 1996; Tan 2004). Opinions still vary as to whether *Blastocystis* is actually responsible for disease in humans, with reports supporting its role in various intestinal diseases (Andiran et al. 2006; Carrascosa et al. 1996; Leelayoova et al. 2004; Levy et al. 1996) and while others dismiss the role of *Blastocystis* as a causal organism in human disease (Chen et al. 2003; Leder et al. 2005; Tungtrongchitr et al. 2004).

Riboprinting is a common method applied in the classification of protozoa. It involves the analysis of small subunit ribosomal RNA gene (SSU rDNA) by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP; Clark 1992). This method was employed for the molecular characterization of *Blastocystis* isolates, allowing the classification of *Blastocystis* isolates to various genotypic groups based on restriction digest patterns of parasite SSU rDNA. There are two major approaches to this endeavor. One involves the amplification of the entire SSU rRNA gene and the subsequent digestion with several restriction endonucleases (Clark 1997; Kaneda et al. 2001;

Rivera et al. 2005; Yoshikawa et al. 2000). Currently, there are ten genotypes, designated Ribodemes, obtained using this approach (Stensvold et al. 2007). The second approach is similar, but only a 1.1-kbp region of the SSU rRNA gene was amplified and the product digested with the restriction endonucleases *Alu* I, *Hinf* I, and *Rsa* I (Böhm-Gloning et al. 1997; Thathaisong et al. 2003). Six genotypes, called subgroups have been described using this method (Böhm-Gloning et al. 1997).

In addition to humans, *Blastocystis* spp. had also been isolated from various animal sources including cockroaches (Zaman et al. 1993), pigs, cattle (Quilez et al. 1995a, b), and reptiles (Teow et al. 1992). Molecular analysis of isolates from animal sources had shown that there exist many *Blastocystis* isolates that were closely related to isolates from animal hosts, suggesting that animals are a potential source of human infection (Abe et al. 2003a, b, c; Noël et al. 2005; Thathaisong et al. 2003; Yoshikawa et al. 1996).

In this paper, we present a prevalence study of *Blastocystis* in Singapore. Fecal samples obtained from the National University Hospital (NUH), a major hospital in Singapore, were surveyed for the presence of *Blastocystis*, and these isolates were subjected to PCR-RFLP analysis. To determine if there were any changes in the prevalent ribotypes in the Singapore population, we compared the ribotypes of the new hospital isolates with ribotypes of *Blastocystis* isolated several years ago in Singapore (Ho et al. 2001). To determine if the possibility of zoonotic spread existed, we also looked at the ribotypes of some *Blastocystis* spp. isolated from a variety of animals in Singapore.

Materials and methods

Isolation of *Blastocystis* from NUH

Approval from the National Healthcare Group Institutional Review Board had been obtained before the commencement of this project. Fecal samples obtained from NUH were cultured in Jones' medium (Jones 1946) supplemented with 10% horse serum (Sigma-Aldrich) and incubated for 3 days at 37°C. Fecal cultures were then subjected to microscopic examination and samples positive for *Blastocystis* were subcultured into fresh Jones' medium with 10% horse serum. Cultures were maintained by subculturing into fresh medium at every 5 days. Details of the isolates used in this study were listed in Table 1. Fecal samples were collected over a period of 6 months.

Blastocystis isolates for comparative studies

Other than clinical samples mentioned above, axenized samples of *Blastocystis* from humans (HB, HC, HE, HG, and HSi) and various hosts (S1 and WR1 from rats; B12 and B16 from reptiles) and non-axenic isolates from cockroaches (C12 and C14) were also used. Axenic cultures were maintained in Iscove's modified Dulbecco's medium (IMDM) with 10% horse serum, while non-axenic isolates were maintained in Jones' medium supplemented with 10% horse serum. Details of the origins and other information of these isolates can be found in Tables 1 and 2.

Table 1 Summary of the information available on human *Blastocystis* isolates and the results of PCR-RFLP analysis on the DNA extracted from these isolates

Isolate	Year isolated	Source	Clinical information	Average size (μm)	Host's age (sex)	SSU rRNA gene Ribodeme (primer set A)	SSU rRNA gene subgroup (primer set B)
H1	2005	NUH	NA	15.4	NA	2	I
H2	2005	NUH	Outpatient	16.3	51(M)	2	I
H3	2005	NUH	Outpatient	22	45(F)	2	I
H4	2005	NUH	Health screening	28.9	30(F)	2	I
H5	2005	NUH	NA	17.4	NA	1	III
H6	2005	NUH	Health screening	22.5	54(F)	2	I
H7	2005	NUH	Heart failure	33.6	60(M)	2	I
H8	2005	NUH	Health screening	18.6	36(F)	2*	I*
H9	2005	NUH	Health screening	15.1	51(F)	1	III
HB	1991	Singapore	NA	NA	NA	10	VI
HC	1993	Singapore	NA	NA	NA	10	VI
HE	1988	Singapore	NA	NA	NA	10	VI
HG	1990	Singapore	NA	NA	NA	10	VI
HSi	1997	Pakistan	NA	NA	NA	3	IV

Isolate H8 closely resemble the other members of subgroup I and Ribodeme 2 (described in Clark 1997) but with a single extra product after digestion by *Rsa I*, and hence was marked with an asterisk.

Table 2 Details of the *Blastocystis* isolates from animals used in this study as well as the results of PCR-RFLP analysis on the DNA extracted from these isolates

Isolate	Year isolated	Source	Host	SSU rRNA gene Ribodeme (primer set A)	SSU rRNA gene subgroup (primer set B)
B12	1993	Singapore	Python	–	New (1B)
B16	1993	Singapore	Red-footed tortoise	–	New (2B)
C12	1993	Singapore	Cockroach	New (1A)	New (3B)
C14	1993	Singapore	Cockroach	New (1A)	New (3B)
S1	1997	Singapore	Rat	3#	IV
WR1	1997	Singapore	Rat	3#	IV

"New" indicates that the riboprint pattern had not been described in previous publications. B12 and B16 produced patterns distinct from one another while C12 and C14 produced identical banding patterns after restriction digest. Results marked with # were also described in Ho et al. (2001). We were not able to amplify isolates B12 and B16 using primer set A and this was denoted by *en dashes*.

Extraction of genomic DNA

Fecal cultures positive for *Blastocystis* were washed five times with phosphate-buffered saline (PBS; Oxoid) at 720 g for 10 min. The pellets were then resuspended in 1 ml PBS and transferred to microfuge tubes and centrifuged at 2,500×*g* for 5 min. The supernatants were then discarded and the pellets stored at −80°C until required for DNA extraction. Extraction of DNA was carried out using the phenol-chloroform method.

PCR-RFLP analysis

The primers (5→3′) used were: primer set A, forward A, GCTTATCTGGTTGATCCTGCCAGTAGT, and reverse A, TGATCCTTCCGCAGGTTCACCTA; and primer set B, forward B, GGAGGTAGTGACAATAAATC, and reverse B, ACTAGGAATTCCTCGTTCATG.

Primer set A was first described by Yoshikawa et al. (2000; referred to as primers SR1F and SR1R) and amplify the entire *Blastocystis* SSU rRNA gene sequence. Primer set B is a modification of the primer set used in Böhm-Gloning et al. (1997) and was designed to amplify a 1.1-kbp region within the *Blastocystis* SSU rRNA gene. The reverse primer used in this study was modified for amplification of SSU rRNA gene from *Blastocystis* isolates from non-human host compared to the original (see Fig. 1). The combination of this new reverse primer and the original forward primer was designated in this study as primer set B. PCR was carried out using either primer sets described above (conditions: 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 54°C for 1 min, and then 72° for 1.5 min. This is followed by a final annealing cycle of 72°C for 10 min. In the case of primer set B, the primer annealing temperature used was 49°C instead of 54°C). The PCR products obtained were subsequently purified using the Qiaquick PCR purification kit (Qiagen) as per manufacturer's instructions.

Purified PCR products were digested with the restriction endonucleases *Alu* I, *Hinf* I, and *Rsa* I (New England Biolabs) in accordance to the manufacturer's recommendations. The digestion products were separated using gel electrophoresis on a 3% agarose gel with Tris/Borate/EDTA buffer and subsequently stained with ethidium bromide and viewed under a UV transilluminator (Bio-Rad).

Results and discussion

Nine *Blastocystis*-positive cultures were isolated from a total of 276 fecal samples taken from NUH. This gives an isolation rate of 3.3%. Cultured in Jones' medium supplemented by 10% horse serum, the predominant form observed was the vacuolar form, with the granular form occasionally seen. We could not differentiate *Blastocystis* isolates from one another using morphological or size measurements nor could we distinguish any differences between ribotypes (Table 1).

PCR-RFLP with primer set A

PCR amplification of the DNA extracted from the nine clinical isolates from NUH (H1–H9) yielded products of approximately 1.8 kbp in size. RFLP analysis of the PCR products using *Hinf* I, *Rsa* I, and *Alu* I demonstrated that the nine *Blastocystis* isolates could be classified into two broad groups based on their restriction profiles (Fig. 2). Six isolates (H1–4, H6, and H7) produced identical *Hinf* I, *Rsa* I, and *Alu* I restriction patterns similar to that described for Ribodeme 2 by Clark (1997). Another isolate H8 produced an almost identical pattern as the six isolates above but with an additional band with molecular weight of approximately 980 bp observed after digestion with *Rsa* I. The two other

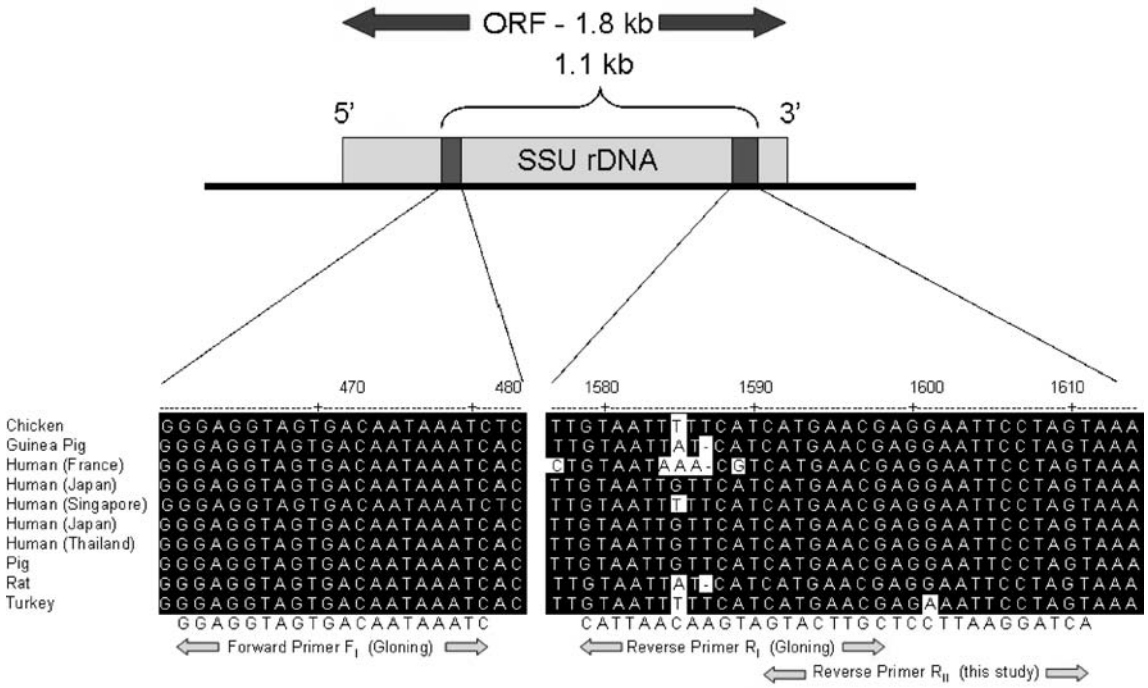

Fig. 1 Alignments between the primer sets reported in Böhm-Gloning et al. (1997) and with primer set B used in this study. Primer set B is a combination of the forward primer used by Böhm-Gloning et al. (1997) with a new reverse primer designed to better amplify DNA of *Blastocystis* isolates from non-human hosts

isolates (H5 and H9) produced banding patterns consistent with Ribodeme 1 as described by Clark (1997).

PCR-RFLP with primer set B

As was previously mentioned, the classification of *Blastocystis* via PCR-RFLP of the SSU rRNA gene can be divided into two approaches. The second, described by Böhm-Gloning et al. (1997), used primers designed to amplify a 1.1-kbp region within the SSU rRNA gene, and the authors had also tested and proven the specificity of these primers against DNA from intestinal bacteria and the intestinal protozoan *Entamoeba histolytica*. In addition, the restriction endonucleases used were standardized (*Alu* I, *Hinf* I, and *Rsa* I), which eased comparison of results between different reports that used the same methodology. PCR of DNA extracted from these nine isolates produced a product of molecular weight 1.1-kbp. RFLP analysis of the nine hospital isolates using *Hinf* I, *Rsa* I, and *Alu* I produced three distinct restriction profiles (Fig. 3). Six of the isolates (H1–4, H6, and H7) produced identical banding patterns and were consistent with subgroup I (Böhm-Gloning et al. 1997; Thathaisong et al. 2003). Two isolates (H5 and H9) produced banding patterns consistent with subgroup III (Böhm-Gloning et al. 1997). Isolate H8 produced a restriction banding pattern similar to the subgroup I isolates, except that an extra 900-bp band was produced after *Rsa* I digestion that was not observed with the members of the subgroup.

The *Blastocystis* prevalence of 3.3% we observed in patients of NUH was comparable to prevalence reported from most developed countries such as England (6.9%; Windsor et al. 2002), Sweden (4%; Svenungsson et al. 2000) and neighboring countries Malaysia (4%; Sinniah et al. 1994; Menon et al. 1999) and Indonesia (6.52%) (Simadibrata et al. 2004). This was lower than the prevalence in the USA, which was recorded at 23% (Amin 2002). While the prevalence in Japan among only native Japanese were lower than Singapore at 0.5%, it was found to be higher at 7.4% among foreign residents in Japan (Horiki et al. 1997).

Two of the isolates (H5 and H9) were mapped by PCR-RFLP (using primer set A) to belong to Ribodeme 1 (Clark 1997). In a study conducted in the Philippines by Rivera et al. (2005), they demonstrated that a majority of human isolates (10/12 or 83.3%) belonged to Ribodeme 1. In Japan, this Ribodeme accounted for 15.6% (10/64) of human isolates tested in a study by Kaneda et al. (2001). Ribodeme 1 is also associated with carriage in animals. Abe et al. (2003a) reported that *Blastocystis* isolates from birds exhibited the same RFLP patterns for *Hinf* I and *Rsa* I, although they used *Hae* III rather than *Alu* I in their study. Similarly, other studies have shown that *Blastocystis* isolates displaying similar RFLP patterns for at least two

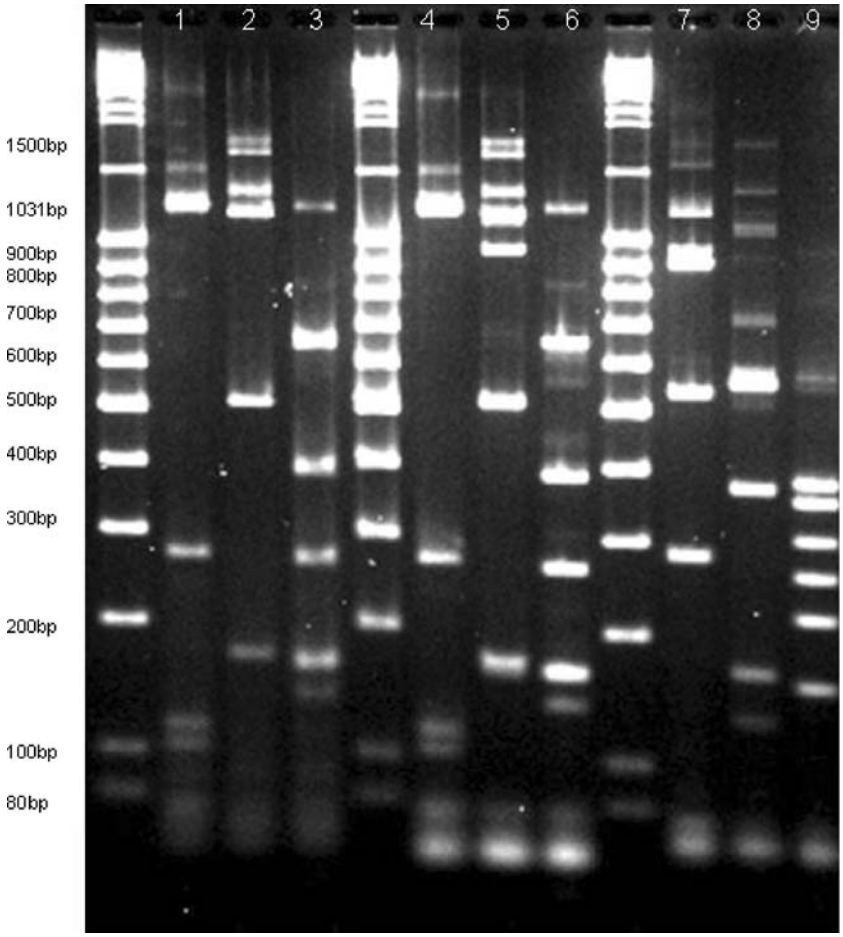

Fig. 2 Result of PCR-RFLP analysis of *Blastocystis* amplified using primer set A. Size markers (Fermentas DNA Ladder SM0403) are run in between the digests of each isolate. The leftmost three digests were the digestions of the PCR product of H1 and is also representative of H2, H3, H4, H6, and H7. The center group of three digests are that of the PCR product of H8 and the rightmost group of digests are that of H5 (an identical pattern is also observed with H9). The PCR products in *lanes 1*, *4*, and *7* were digested by *Hinf* I; the PCR product in *lanes 2*, *5*, and *8* were digested with *Rsa* I; and the PCR product in *lane 3*, *6*, and *9* were digested with *Alu* I

of the restriction enzymes used in our study could be found in pigs and cattle (Abe et al. 2003b) and primates (Abe et al. 2003c). This would suggest that Ribodeme I of *Blastocystis* can be found in a variety of different hosts, and zoonotic transmission likely occurs between humans and animals. When amplified by primer set B and subsequently digested by the restriction enzymes *Alu* I, *Hinf* I and *Rsa* I, the PCR-RFLP profiles of H5 and H9 isolates were matched to ribosomal subgroup III, first described by Böhm-Gloning et al. in 1997. Subgroup III was found to be the most commonly occurring (138/153 or 90.2%) *Blastocystis* subgroup isolated from humans in Thailand (Thathaisong et al. 2003). More interestingly, the same study found that all the *Blastocystis* isolates that from pigs and also one from a horse were also classified into subgroup III. In addition, the horse isolate and one of the pig isolates were shown via sequence and phylogenetic analysis to be monophyletic and closely related to *Blastocystis* isolated from humans (92–94% identity; Thathaisong et al. 2003).

The remaining six isolates (H1, H2, H3, H4, H6, and H7) were mapped to Ribodeme 2 (Clark 1997) using primer set A. An isolate H8 was almost completely identical except that there existed a polymorphism that created an extra *Rsa I* cut site. Isolates from Ribodeme 2 made up the majority (7/9 or 77.8%) of the isolates from NUH. Ribodeme 2 isolates also formed the majority in a study in Japan (Kaneda et al. 2001), where 45% (or 29/64) of all isolates tested were from this ribodeme. This, however, contrasted to the results from the Philippines by Rivera et al. (2005) where no human isolates were found to belong to this ribodeme, although the majority (83.3%) of human isolates belonged to Ribodeme 1, which was also a genotype observed in the current study. Isolates from this Ribodeme had also been discovered among isolates from birds (Abe et al. 2003a), cattle, pigs (Abe et al. 2003b), and

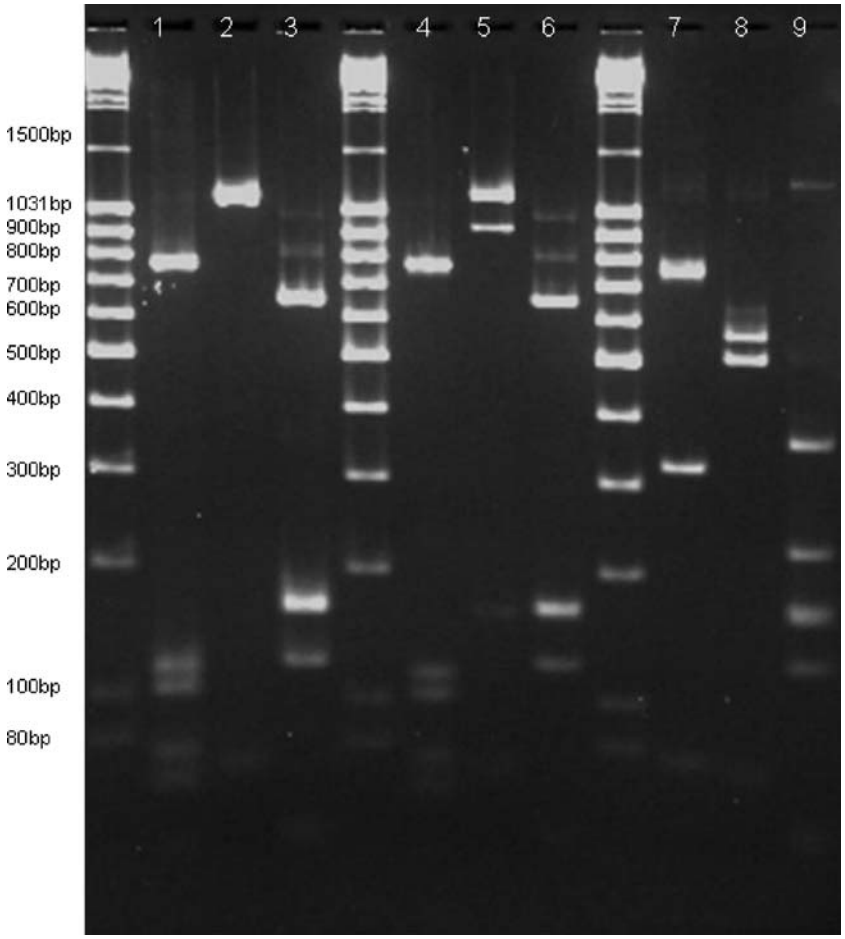

Fig. 3 Result of PCR-RFLP analysis of *Blastocystis* amplified using primer set B. Size markers (Fermentas DNA Ladder SM0403) are run in between the digests of each isolate to allow for easy separation and size determination. The *leftmost* three digests were the digestion of the PCR product of H1 and is also representative of H2, H3, H4, H6, and H7. The center group of three digests are that of the PCR product of H8 and the *rightmost* group of digests are that of H5 (an identical pattern is also observed with H9). The PCR products in *lanes 1*, *4*, and *7* was digested by *Hinf* I; the PCR product in *lanes 2*, *5*, and *8* was digested with *Rsa* I; and the PCR product in *lane 3*, *6*, and *9* was digested with *Alu* I

from primates (Abe et al. 2003c). When the seven isolates were amplified using primer set B, they could be mapped to subgroup I as reported by Böhm-Gloning et al. (1997) and Thathaisong et al. (2003). Six isolates belonged to the same subgroup, while isolate H8 was very closely related, distinguished only by a polymorphism that gave an extra *Rsa I* cut site, similar to that observed when primer set A was used.

There exists a confusing array of methods to classify *Blastocystis* isolates. Recently, a consensus in terminology for *Blastocystis* subtypes was described (Stensvold et al. 2007). In accordance with this consensus, Ribodeme 1/subgroup III isolates from NUH would be classified as *Blastocystis* sp. subtype 1, while the isolates matched to Ribodeme 2/subgroup I would be classified as *Blastocystis* sp. subtype 3. This agreement between Ribodeme, subgroups, and subtypes indicates that the genotyping from different studies were robust. Interestingly, subtype 3, which comprised the majority (78%) of isolates in the present study, was suggested to be a subtype of human origin, while the remaining subtypes were zoonotic (Noël et al. 2005; Yoshikawa et al. 2004). The results of the current study are similar to a survey of *Blastocystis* subtypes from Japan, Bangladesh, Pakistan, Germany, and Thailand (Yoshikawa et al. 2004), where the predominant ribotype was subtype 3 (41.7–92.3%), followed by subtype 1 (7.7–25.0%) or subtype 4 (10.0–22.9%). The predominance of subtype 3 in an urbanized city state such as Singapore where there is limited opportunity for zoonotic transmission further reinforces the idea that this subtype is of human origin. Previous reports on the occurrence of subtype 3 in a variety of animals suggest that humans are reservoirs for animal infection.

We then looked at four human *Blastocystis* isolates (HB, HC, HE, and HG) and six animal isolates (B12, B16, C12, C14, WR1, and S1) previously isolated in Singapore. For comparison, we also looked at another human *Blastocystis* isolate from Pakistan (HSi) that was in our collection. The

Singapore human isolates were shown by Ho et al. (2001) to produce identical PCR-RFLP profiles that matched Ribodeme 10. These isolates were further mapped by Yoshikawa et al. (1998) and Noël et al. (2005) to subtype 2 according to PCR analysis using STS primers. We tested all the above isolates using primer set A and found that none of the human or animal isolates produced a restriction pattern matching any of the hospital isolates (Tables 1 and 2). We were unsuccessful in our attempts to amplify reptilian isolates B12 and B16 using primer set A. No PCR-RFLP analysis using primer set B had previously been done with these isolates. We carried out PCR-RFLP analysis with this primer set, and the resulting profile of the human isolates HB, HC, HE and HG were matched to subgroup VI (Thathaisong et al. 2003; results not shown) while HSi was matched to subgroup IV. PCR-RFLP of the animal isolates using primer set B showed that *Blastocystis* from cockroaches (C12 and C14) and reptiles (B12 and B16) displayed novel profiles that were not previously described in literature (results not shown), while the rodent isolates matched subgroup IV, which consists of zoonotic isolates (results not shown; Noël et al. 2005; Table 2). Interestingly, human isolate HSi was also found to belong to this subgroup, further reinforcing the zoonotic potential of this genotype. None of the hospital isolates in the present study were of a similar ribotype to the human and animal isolates used for comparison in this study. However, with only nine samples, it is difficult to draw firm conclusions on the possibility of zoonotic spread or evolving genotypes between the current and previous studies.

In conclusion, a survey of stool samples from NUH revealed that the carriage rate of *Blastocystis* was approximately 3.3%. We were not able to differentiate these isolates from one another by morphological criteria. We were able to separate, using PCR-RFLP, these isolates into two distinct groups based on their ribotypes. The predominant genotype in Singapore is subtype 3, followed by subtype 1, which supports the idea that subtype 3 is a *Blastocytis* genotype of human origin.

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