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Short Communication

Genetic diversity of rodent *Blastocystis* sp. from Peninsular Malaysia

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Abstract. Rodents are ubiquitous zoonotic vectors for many human pathogens including *Blastocystis* sp. In this study, we examined the prevalence and subtypes of *Blastocystis* sp. in rodents captured from Peninsular Malaysia. A total of 293 rodents predominantly brown rat (*Rattus norvegicus*) (290 of 293, 99.0%) and house shrew (*Suncus murinus*) (3 of 293, 1.0 %), were captured in the vicinity of popular eateries in two cities (Kuala Lumpur and Ipoh) in Peninsular Malaysia. *In vitro* cultivation method showed presence of *Blastocystis* sp. in approximately half (133 of 290, 45.9%) of the brown rats tested. Among the 47 *Blastocystis* isolates subtyped using partial small subunit ribosomal RNA gene analysis, ST4 was the most abundant (43 of 47, 91.5%) followed by ST1 (2 of 47, 4.3%), ST5 (1 of 47, 2.1%) and ST7 (1 of 47, 2.1%). Our findings highlighted the importance of rodents as a source of *Blastocystis* sp. infection in Malaysia and showed the high prevalence of ST4 within the rodent population infected with *Blastocystis* sp.

Blastocystis sp. is a single-celled intestinal parasite belonging to an extremely diverse group of protists called Stramenopiles that colonizes a large range of vertebrates and invertebrates (Scanlan and Stensvold, 2013). This parasite is estimated to colonize between 1 and 2 billion people worldwide (Scanlan and Stensvold, 2013). Despite its common presence in human, the clinical significance of *Blastocystis* sp. is a matter of intense discussion while effective therapy remains elusive (Stensvold *et al.*, 2010). Majority of patients infected with *Blastocystis* sp. were asymptomatic and for symptomatic patients, most frequent signs include diarrhea, abdominal pain and cutaneous manifestations (Salvador *et al.*, 2016).

The genus *Blastocystis* sp. can be classified into 17 small subunit ribosomal RNA (SSU-rDNA) lineages known as subtypes (STs) (Alfellani *et al.*, 2013b). Surveys of *Blastocystis* sp. ST prevalence in different countries showed significant variation between human populations (Alfellani *et al.*, 2013a). Collectively, ST1 to ST4 accounted for approximately 92.8% of human infection while the remaining 7.2%

comprised of ST5 to ST9 (Alfellani *et al.*, 2013a). Analysis of *Blastocystis* sp. ST in the animal population has expanded the host and geographic range of several STs and importantly suggested that livestock is not a major contributor to human infection (Alfellani *et al.*, 2013b).

In Malaysia, *Blastocystis* sp. was found to contaminate the rivers therefore leading to significant waterborne infections especially in rural areas where access to clean water was limited (Abdulsalam *et al.*, 2012; Ithoi *et al.*, 2011; Noradilah *et al.*, 2016). Molecular analysis showed the presence of multiple *Blastocystis* sp. subtypes in river water samples and ST3 was found to be the most robust and resistant subtype in the environment (Noradilah *et al.*, 2016). The same subtype was also implicated in causing intestinal infection particularly in rural schoolchildren (Nithyamathi *et al.*, 2016). In animals, *Blastocystis* sp. was previously reported in pig-tailed macaque, Sumatra Orang Utan, ostriches, water monitor lizard and mouse-deer (Lim *et al.*, 2008; Chandrasekaran *et al.*, 2014; Mohd Zain *et al.*, 2017).

Rodents are notorious for their tendency to contaminate public places with their urine and faeces while serving as reservoir host to a variety of human pathogens such as *Leptospira* sp. (Benacer *et al.*, 2016). Therefore, our aims in this study were to determine the prevalence and subtypes of *Blastocystis* sp. in rodent population in Malaysia. Our findings will provide evidence for the role of rodents in the transmission of *Blastocystis* sp. as well as the subtypes that are circulating locally.

All animals used in this study were handled according to Institutional Animal Care and Use Committee (IACUC), University Malaya guidelines (Reference No.: ISB/31/01/2013/SNMZ (R)). A written permission to conduct the study was obtained from the Ipoh City Council and Kuala Lumpur City Hall. The sampling area is not privately-owned and the field studies did not involve endangered or protected species.

Rodents were caught between June 2013 and August 2014 at the vicinity of public spaces such as restaurants and wet markets. Sampling was carried out in two urban cities namely Ipoh (4.5975° N, 101.0901° E), in the state of Perak and Kuala Lumpur (3.1333° N, 101.6833° E), the capital city of Malaysia, using convenient sampling method incorporating wire-box traps with selected baits.

Rodents were euthanized using chloroform, their caecums were removed and its contents were cultured using *in vitro* cultivation method (Suresh and Smith, 2004). The positive isolates were fixed with methanol and stained with 10% Giemsa stain to observe the detailed morphology of the protozoan. Samples were considered negative if *Blastocystis* sp. were not observed when cultured up to day 5 at 37°C. After two subcultures *Blastocystis* isolates were harvested, spun and the suspension stored at -20°C prior to DNA extraction.

Genomic DNA was extracted using the Qiagen stool extraction kit following the manufacturer's instructions. Samples were then subjected to DNA barcoding method as previously described (Mohd Zain *et al.*, 2017). The sequences obtained were queried into both *Blastocystis* PubMLST (https://pubmlst.org/blastocystis/) and NCBI (http://www.ncbi.nlm.nih.gov/BLAST) databases. Phylogenetic tree was constructed with MEGA v6.06 using maximum likelihood method and Kimura 2-parameter model (Tamura *et al.*, 2013). Branch reliability was assessed using bootstrap analysis of 1000 replicates. Three reference sequences for each ST1-7 were obtained from *Blastocystis* PubMLST database and were included in the phylogenetic tree analysis alongside the local samples.

A total of 293 rodents were captured comprising of brown rat (*Rattus norvegicus*) (290 of 293, 99.0%) and house shrew (*Suncus murinus*) (3 of 293, 1.0%) (Table 1). *Blastocystis* sp. was detected in nearly half (133 of 290, 45.9%) of the brown rats while all three house shrews were negative for *Blastocystis* sp. The rate of infection for rodents captured in Kuala Lumpur and Ipoh was 44.8% (95 of 212) and 46.9% (38 of 81), respectively.

Table 1. The distribution and infection status of rodents captured in Kuala Lumpur and Ipoh

Characteristics	Sampling Sites, n (%)		Total, n
	Kuala Lumpur	Ipoh	
Host species			
Rattus norvegicus	212 (73.1)	78 (26.9)	290
Suncus murinus	0 (0.0)	3 (100.0)	3
Infection status			
Positive	95 (71.4)	38 (28.6)	133
Negative	116 (72.5)	44 (27.5)	160

Table 2. Subtypes of *Blastocystis* sp. from rodents in this study

Blastocystis sp. subtype	No. of isolates, n (%)
ST4	43 (91.5)
ST1	2 (4.3)
ST5	1 (2.1)
ST7	1 (2.1)

Subtypes were successfully determined in 47 (35.3%) of the 133 positive samples (Table 2). The predominant subtype was ST4 (43 of 47, 91.5%) followed by ST1 (2 of 47, 4.3%), ST5 (1 of 47, 2.1%) and ST7 (1 of 47, 2.1%). In the remaining 86 positive samples, PCR yielded faint bands which resulted in failure during DNA sequencing. Therefore, these samples were considered untypeable.

Phylogenetic analysis showed clustering of local samples according to reference STs (Figure 1). Assignment of STs by phylogenetic tree was consistent with that obtained by BLAST queries at *Blastocystis* PubMLST database. However, one sample (R22) identified as ST7 was not included in the phylogenetic analysis because the complete partial sequence could not be obtained due to presence of mixed signals in the middle of the sequencing result. Approximately 236bp clean sequencing result was recovered for R22 which was used for BLAST analysis leading to identification of ST7 for this isolate.

In this study, we provided the first evidence of the high carriage rates of *Blastocystis* sp. among local rodent population. Brown rats were known reservoirs of zoonotic diseases, carrying important human pathogens such as *Escherichia coli*, *Clostridium difficile*, and *Salmonella enterica* (Firth *et al.*, 2014). Our findings expanded the range of organisms carried by brown rats therefore stressing the importance of eradicating point that efforts to eradicate rodent populations in urban areas even more important.

Our study confirmed that ST4 is the most dominant subtype in Malaysian rodent population. This subtype was also isolated from various animal hosts such as ostrich, deer, snow leopard, kangaroo and wallaroo (Roberts *et al.*, 2013). In human population ST4 was commonly found across Europe but it was rarely detected in other continents (Alfellani *et al.*, 2013a; Forsell *et al.*, 2012). This discrepancy could be attributed to the methodology used for *Blastocystis* subtyping. Studies utilizing the Sequence-Tagged-Site (STS) PCR were proven to underdiagnose ST4, possibly due to the presence of substantial genetic variation in the target loci (Stensvold, 2013).

Other STs namely, ST1, ST5 and ST7 were also identified in Malaysian rodent population. ST1 is the most dominant human subtype in many countries such as Thailand (Leelayoova *et al.*, 2008; Jantermtor *et al.*, 2013), China (Li *et al.*, 2007), Germany (Böhm-Gloning *et al.*, 1997), Greece (Menounos *et al.*, 2008), and Singapore (Wong *et al.*, 2008). In Malaysia, ST1 was shown to be the second most common subtype among colorectal cancer (CRC) patients (Kumarasamy *et al.*, 2014) and schoolchildren (Nithyamathi *et al.*, 2016).

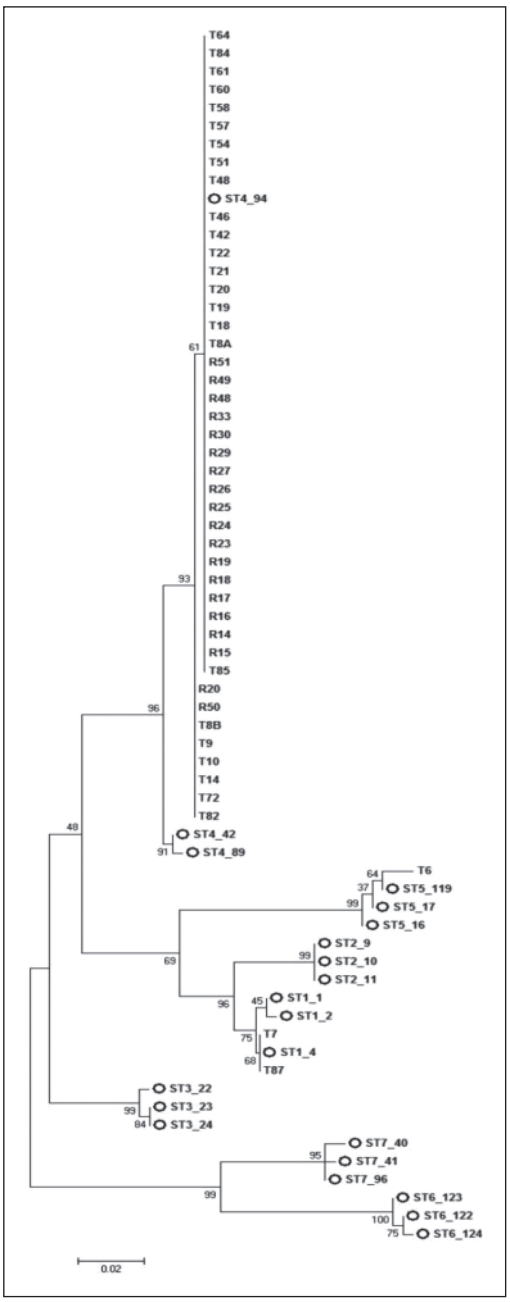

Figure 1. Phylogenetic tree of the partial small subunit rRNA sequences of *Blastocystis* isolates from rodents as well as reference sequences from *Blastocystis* PubMLST database, indicated by the open circle symbol.

A wide range of animals were carrying *Blastocystis* ST1 particularly monkeys, chimpanzees, cattles, pigs, horse, ostriches, dogs, goats and chickens (pheasant) (Yoshikawa *et al.*, 2003; Navarro *et al.*, 2008; Tan *et al.*, 2013; Wang *et al.*, 2013; Ruaux and Stang, 2014). Zoonotic transmission was also reported for this subtype from farm animals (Noël *et al.*, 2003). ST5 was commonly found in pigs (Wang *et al.*, 2014) and domestic animals i.e cattle (Alfellani *et al.*, 2013b) whereas ST7 has been reported in chickens, quails, geese, birds and goats. However, both subtypes were rarely found in human surveys (Abe *et al.*, 2002; Noël *et al.*, 2003; Alfellani *et al.*, 2013a; Tan *et al.*, 2013). The presence of mixed signal for ST7 in this study was probably due to intragenomic variation between 38 SSU rRNA gene copies within the genome (Meloni *et al.*, 2012).

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