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Research Note

Morphological and molecular detection of *Blastocystis* in wildlife from Tioman Island, Malaysia

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Abstract. *Blastocystis* infection is widely reported in wildlife, livestocks and in non-human primates however, occurrence in Malaysian wildlife is scarce. A wildlife survey on Tioman Island captured six water monitor lizard (*Varanus salvator*), four mouse-deer (*Tragulus* sp.) and one Malayan porcupine (*Hystrix brachyura*) based on convenience sampling. Intestinal contents from each animal were subjected to *in vitro* cultivation method using Jones medium supplemented with 10% horse serum. Low prevalence of infections was detected with only 1/6 (16.7%) water monitor lizard and 1/4 (25%) mouse-deer infected. The vacuolated form was the most common cell form found in both cultures with similar morphology to *B. hominis*. However, the monitor lizard isolate propagated well in the laboratory for several months using Jones medium while mouse-deer isolate could not be maintained for more than a week. The reptilian isolates grew optimally at a lower temperature of 24ºC compared to 37ºC for the mouse-deer isolate. Using the DNA barcoding method, both isolates were confirmed to be *Blastocystis* sp. Sequence obtained from a monitor lizard isolate has 94% sequence identity to *B. lapemi*, an isolate recovered from a reptile sea-snake whereas a mouse-deer isolate has 99% sequence identitical to *B. hominis* HJ01-7. The phylogenetic tree revealed that the monitor lizard isolate were positioned within the herptiles clade (clade VIII) while the mouse deer isolate located at the homoithermal clade (clade IV). The present paper is the first report on the presence as well as genetic characteristics of *Blastocystis* in wildlife captured from Tioman Island, Pahang.

Humans isolates are known as *B. hominis* (Brumpt, 1912) while non-human isolates are termed *Blastocystis* sp. as most animal isolates have not been genetically analyzed (Stenzel and Boreham, 1996; Yoshikawa *et al.*, 2004a).

Due to the general morphological similarities among the different isolates and polymorphism within isolates, other criteria, such as optimal growth temperature, karyotypic profiles and gene sequences have been employed to differentiate isolates of different hosts. Only a handful of isolates from specific hosts are successfully published namely; *B. ratti* for rats (Chen *et al.*, 1997), *B. galli* for chickens (Belova, 1998), *B. anatis* for domestic ducks (Belova, 1991), *B. anseri* for domestic geese (Belova,

1992), *B. lapemi* for sea snakes (Teow *et al.*, 1991) and other reptiles including a reticulated python (*B. pythoni*), red-footed tortoise (*B. geocheloni*), and rhino iguana (*B. cycluri*) (Singh *et al.*, 1996). The extensive genetic diversity of this organism, even among isolates from one host, makes the host-specific naming of species misleading. The degree of genetic divergence between the major clades seen in *Blastocystis* would be considered sufficient on its own to justify separate species names for each. However, the more appropriate nomenclature proposed by Stensvold *et al.* (2007) that all mammalian and avian isolates should be designate as *Blastocystis* sp. subtype *n* where *n* is a number from 1 to 17.

Despite many zoonotic isolates identified from various mammals, birds and reptiles (Clark, 1997; Abe *et al.*, 2003; Arisue *et al.*, 2003) worldwide, Lim *et al.* (2008) reported *Blastocystis* sp infecting wildlife animal particularly pig-tailed macaque and Sumatra Orang Utan in a Malysian zoological garden and large-billed crows (*Corvus* spp.) in Bangsar, Kuala Lumpur (Lee, 2008). While Tan *et al.* (2013) successfully determined the occurrence and genetic diversity of *Blastocystis* sp. in goats obtained from five different farms in Serdang, Selangor. *Blastocystis* sp. infected 30.9% of goats primarily with ST1, followed by ST7, ST6 and ST3 subtypes.

To date, there are gaps of information on the status, morphology as well as genetic diversity of *Blastocystis* sp. from wildlife in this country. The hypothesis that intimate association between human and animals could facilitate transmission, led us to investigate the occurrence of *Blastocystis* especially in wildlife captured from Tioman Island which is one of the main tourist spots located along the eastern coast of Malaysia.

Tioman Island is situated approximately 56km off the east coast of peninsular Malaysia and is part of the state of Pahang and is gazzetted as one of the National Marine Parks of Peninsular Malaysia.

Sampling was conducted from 27th to 29th August 2013, in the surrounding area of Kampung Salang, Tioman Island, Malaysia (N: 2.875836, E: 104.154144) by adopting a convenience sampling method in which the animals were captured based on convenience, accessibility and availability. A total of 11 wild animals consisting of six water monitor lizards (*Varanus salvator*), four mouse-deer (*Tragulus* sp.) and a Malayan porcupine (*Hystrix brachyura*) were captured close to the vicinity of human habitation with the assistance of members from the Department of Wildlife Malaysia (PERHILITAN).

Necropsy was performed immediately after killed with a single gun shot to avoid autolysis. *Post mortem* investigation was performed to remove the digestive tract from the body cavity and fresh faecal samples were collected either by cutting open the digestive tract or squeezing the contents out of the tract into the collection container through the large intestine (Farris *et al.*, 2013).

Approximately 2g of each faecal sample was inoculated into a sterile screw-top containing 3 ml of Jones medium supplemented with 10% heat-activated horse serum (Jones, 1946; Suresh and Smith, 2004). Each sample was incubated at 37^{o}C for 48 to 72 hours. Positive samples were subsequently maintained by sub-culturing for staining and were kept at -20°C for polymerase chain reaction (PCR).

The positive faecal smears were fixed with methanol and stained with 10% Giemsa stain to observe the detailed morphology of the protozoan. The stained specimens were observed under microscopy at 400X and 1000X magnification and were analyzed according to the shape and size of the microorganism.

Faecal DNA extraction was performed using the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. The *Blastocystis*-specific primer, BhRDr (GAGCTTTTTAACT GCAACAACG; Scicluna *et al.*, 2006) and the broad-specificity eukaryote-specific primer, RD5 (ATCTGGTTGATCCTGCCAGT; Clark, 1997) were used in a single step PCR reaction to amplify a 600 bp region of the SSU rRNA gene. Amplification of 5 µl genomic DNA was

carried out in 25 µl reaction containing 1.0mM of the dNTPs, 0.5mM of each primer, 1 x PCR buffer, 2.5mM MgCl and 1 U Taq DNA Polymerase (recombinant) (FERMENTAS, USA). Amplification conditions consisted of an initial denaturation at 94°C for 1 min, 30 cycles each of 94°C for 1 min, 59°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 2 min (Thermo Cycler-Bio-Rad). The amplification products were then electrophoresed through 1.5% agarose gels (PROMEGA USA) in Tris-Acetate-EDTA (TAE) buffer. Gels were stained with SYBR ® Safe DNA gel stain and visualized using ultra-violet gel documentation system (Uvitec, United Kingdom). The DNA fragment size was estimated using a 100bp ladder. PCR products of approximately 600bp were sent to First Base Laboratories Sdn. Bhd. for purification and sequencing.

The SSU rDNA sequences were then compared to those available in the GenBank database using the BLAST (Basic Local Alignment Search Tool) program run on the National Centre for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/BLAST) and the *Blastocystis* Sequence Typing website (http://www.publmst.org/blastocystis) (Scicluna *et al.*, 2006; Roberts *et al.*, 2013). Sequences were deposited in GenBank with the accession numbers KX908212 and KX908213.

The sequences were assembled using Seqman (DNAStar Lasergene, USA) and were then analyzed using BioEdit program version 7.0 and the ClustalW multiple alignment method. Phylogenetic tree was constructed with MEGA v6.06 using neighbour-joining (NJ) method and Kimura 2 parameter model (Tamura *et al.*, 2011). The principle of neighbor-joining method is to find pairs of operational taxonomic units (OTUs) that minimize the total branch length at each stage of clustering of OTUs starting with a starlike tree (Saitou and Nei, 1987). The selected isolates in this study together with other *Blastocystis* sequences from the GenBank until March 2016; representative of different subtypes were included for phylogenetic analysis (GenBank, NCBI). *Proteromonas lacerate* (U37108) was used as the out-group in view of its close relationship with *Blastocystis* in previous phylogenetic studies (Silberman *et al.*, 1996).

Out of 11 wildlife hosts examined, one monitor lizard (16.7%) and one mouse-deer (25%) were found positive for *Blastocystis* sp. The Malayan porcupine was free from *Blastocystis* infection.

Both isolates were maintained in Jones medium at 24°C (room temperature) and/or 37°C. Culture from the monitor lizard grew optimally at room temperature with a high number of cells recovered. Meanwhile, the mouse-deer isolates only grew in low cell numbers at 37°C. The growth of the mammalian isolate also appeared slower and less consistent than the monitor lizard isolate. The mouse-deer isolate lasted only up to 7 days in culture compared to 30 days for the reptilian isolate.

Apart from *Blastocystis*, presence of another protozoan parasite *Trichomonas* sp. was also detected in both cultures. Noticeably, *Trichomonas* sp. in the mouse-deer isolate had a higher parasite count than *Blastocystis* sp.

The morphology of *Blastocystis* isolates from the water monitor lizard under light microscope was similar to the human isolate, *B. hominis*. The vacuolated form was dominant and spherical in shape with diameter between 6 to 20 µm. This form was characterized by a large central vacuole that occupied the central part of the cell (Fig. 1A).

Faecal culture from mouse-deer contained low numbers of vacuolar forms of *Blastocystis* sp. and range in size between 7 to 23 µm in diameter. The multi-nucleated cells with one or two nuclei located at opposite poles of the small cells were occasionally observed in this isolates (Fig. 1B).

Using the DNA barcoding method, DNA sequences covering the first 500bp (5'-end) of the *Blastocystis* small subunit (SSU) rRNA gene were obtained. The final sizes of the sequences were 548bp and 547bp for both monitor lizard and mouse-deer isolates, respectively. Sequence obtained from monitor lizard isolate showed 94% sequence identity to *B. lapemi* whereas a mouse-deer isolate showed 99% sequence identical to *B.*

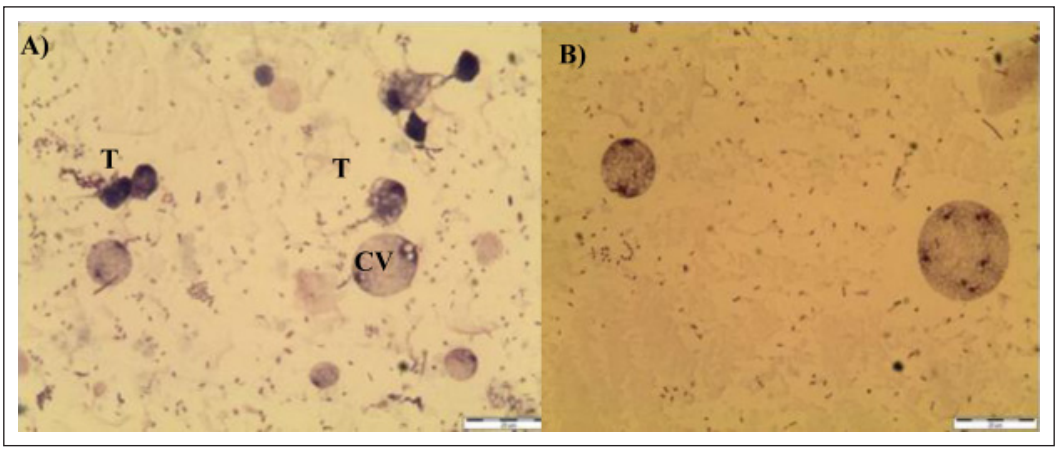

Figure 1. *Blastocystis* sp. isolated from A) water monitor lizard and B) mouse-deer. *Abbreviations: CV*, central vacuole; *T*, *Trichomonas* sp.

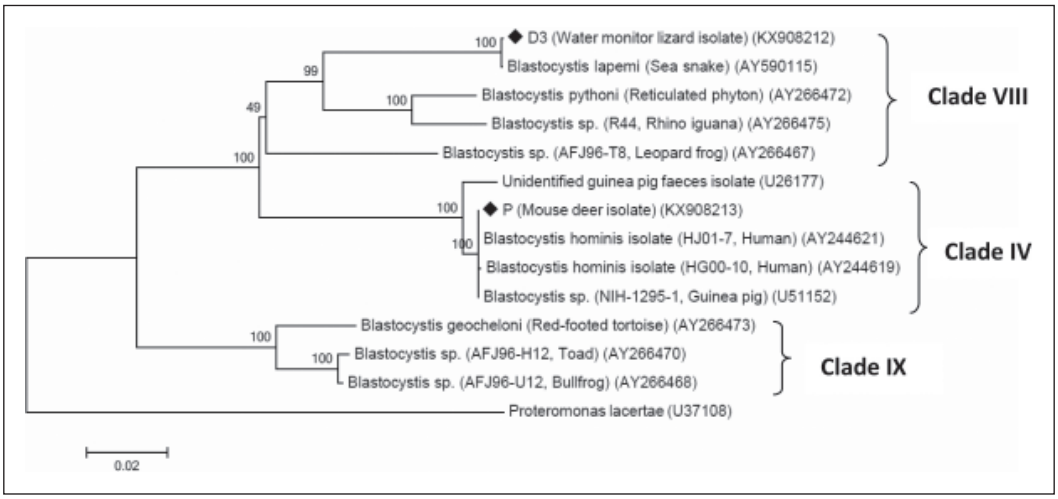

Figure 2. Phylogenetic tree of the small subunit rRNA sequences of *Blastocystis* sp. isolates from monitor lizard and mouse-deer captured in Tioman Island as well as reference sequences from GenBank. The tree was rooted on *Proteromonas lacertae*. Tree construction was done using the neighbour joining method with 1,000 replicates. *Blastocystis* isolates obtained from Tioman Island are in bullet marked. Information on the isolates, hosts, and accession numbers are given in parentheses. Scale bars, 0.02 substitutions (corrected) per site.

hominis HJ01-7. The low sequence identity for the monitor lizard isolate indicated the possibility of a new subtype for this isolate.

The phylogenetic tree of the *Blastocystis* lineage based on the SSU rRNA sequences and inferred by the NJ method comprised of 13 isolates of *Blastocystis*, including the two isolates from wildlife animals captured from Tioman Island and *Proteromonas lacerate* (Fig. 2). Yoshikawa *et al.* (2004b) revealed the presence of ten *Blastocystis* clades in which the monitor lizard isolate were positioned with the herptiles clade (clade VIII) while the mouse deer isolate were positioned within the homoithermal clade (clade IV).

There are several reports of *Blastocystis* sp. infections in domestic and wildlife animals in Malaysia. Suresh *et al.* (1996) reported this parasite in a range of host

including; laboratory animals, sheep, rabbits, monkeys, dogs and cats however, the study was limited to a small number of samples. Similarly, Lim *et al.* (2008) recorded this parasite in pig-tailed macaque and Sumatra Orang Utan while Lee (2008) reported in crows (*Corvus* spp.). Tan *et al.* (2013) reported that 30.9% goats examined were positive for this parasite. More recently, Hemalatha *et al.* (2014) screened a large number of animals in which a total of 104 out of 302 animals (34.44%) were infected with this anaerobic parasite. Moderate infection were observed in the ruminant livestock group (34.5% in cattle; 28.6% in deers, 30% in gaurs; 65% in goats and 57.9% in sheeps) whereas in mammals, the prevalence rate varied around 50% of orang utan and 100% of pigs while in horses and chimpanzee, the organism was undetectable. All the ostriches were positive however, companion animals (i.e cats and dogs), laboratory animals (i.e mice, rats, guinea pigs and rabbits) and all wildlife specimens (i.e black panther, lion, tiger, elephants, tapir, camel, terrapins and wild birds) were completely free from the infection.

This is the first attempt to screen *Blastocystis* in porcupine however the only one sample obtained and was free from infection. Apart from that, this study reported for the first time microscopic details of *Blastocystis* sp. isolates from one of each monitor lizard and mouse-deer. The vacuolar form was the predominant cell type seen in both *in-vitro* cultures as also reported by Tan (2004). Both isolates did not show any differences in morphology and were similar in size ranging between 6-23µm. However, differences were observed in the cultivation temperature where the reptilian isolate propagated well in lower temperature than the mammalian isolate. The distinct difference of the optimal growth temperatures of *Blastocystis* was also previously reported in other reptilians such as tortoises *(Geochelone elephantopus*, *G. elegans* and *G. carbonaria)*, snakes *(Boiga dendrophilla*, *Python reticulatus* and *Elaphe radiata)*, crocodile *(Crocodylus porosus)* and iguana lizard *(Cyclura cornuta)* (Teow *et al.*, 1992).

Jones medium supplemented with 10% horse serum propagated the growth of the reptilian isolates but not the mouse-deer. The growth of the mammalian isolate also appeared slower and less consistent than monitor lizard isolate. The mouse-deer isolate lasted only up to 7 days in culture while isolate from the reptilian was viable for more than 30 days. The presence of *Trichomonas* sp. was also detected in water monitor lizard and mouse-deer isolates with higher counts in mouse-deer than *Blastocystis* sp. The presence of this organism was thought to suppress the growth of *Blastocystis*.

Blastocystis infection in mouse-deer was previously reported in UK by Alfellani *et al.* (2013) in which the derived subtype was ST13. Interestingly, the Malaysian mouse-deer isolate showed high similarity with *B. hominis* suggesting the close proximity interaction between this mammal and human habitation may have resulted in cross-contamination. This result also provided evidence that this organism showed low host specificity as well as highlighting possible transmission risk to humans.

In contrast, DNA barcoding of the isolate from the lizard monitor showed similarity with *B. lapemi* commonly found in sea snakes (*Lapemis hardwickii*) (Teow *et al.*, 1991). Light microscopy showed resemblance to *B. hominis*, however on detailed investigation of the genomic DNA, distinct karyotypic pattern was observed. Teow *et al.* (1992) reported that *Blastocystis* infections were common in reptiles housed at the Singapore Zoological Gardens with eight of the 23 reptilian species screened positive for this protozoan parasite.

Phylogenetic analysis showed that the monitor lizard isolate in this study was related to poikilothermal animal while the mouse-deer isolate clustered together with human and guinea pig isolates. It is evident that mouse deer isolate was closely positioned with those of human origins, suggesting zoonotic potential. Species of the genus *Blastocystis* must not be identified and designated based on host origins (Yoshikawa *et al.*, 2004b). Even though *Blastocystis*

isolates from humans and other animals are morphologically indistinguishable, they showed extensive genetic diversity.

This study provided for the first time morphological and molecular detection of *Blastocystis* sp. in water monitor lizard and mouse-deer in Malaysia. The small sample size may not reflect the true representation of the *Blastocystis* population and subtypes. Therefore, further studies are required to support and extend the current findings on *Blastocystis* species and subtypes in Malaysian wildlife population.

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