MOLECULAR EPIDEMIOLOGY OF *BLASTOCYSTIS* SP IN ANIMALS REARED BY THE ABORIGINES DURING WET AND DRY SEASONS IN RURAL COMMUNITIES, PAHANG, MALAYSIA

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Abstract. In endemic areas of intestinal parasitic infections, prevalence of *Blastocystis* sp in animals has not been clearly elucidated. This is the first study of the distribution of *Blastocystis* sp subtypes in animals reared by Orang Asli population in Pahang, Malaysia during a wet and dry season. Fecal samples of dogs, chickens, goats, ducks, swans, birds and cows were collected and subjected to PCR amplification and sequencing of *Blastocystis* small subunit rDNA. Of 127 fecal samples collected during the wet season, 9% were positive for *Blastocystis* sp, with *Blastocystis* sp ST3 being predominant (16%) followed by ST1 (4%), ST7 (3%), ST4 (2%), ST10 (2%), ST6 (1%), and ST9 (1%). Of 146 fecal samples collected during the dry season 37% were positive, with *Blastocystis* sp ST3 being predominant (10%) followed by ST1 (8%), ST7 (6%), ST4 (5%), ST8 (3%), ST2 (1%), ST6 (1%), ST9 (1%), and ST10 (1%). High prevalence of *Blastocystis* sp was observed in dogs and chickens which carried a diverse range of subtypes especially during the dry season. Dogs and chickens might comprise a part of the transmission dynamics of the infection in the population. Health education related to awareness of hygienic practice and disposal of animals waste should be regularly provided and monitored to prevent the transmission of *Blastocystis* sp infection in this population.

Keywords: *Blastocystis*, aborigine, animal, dry season, wet season, Malaysia

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INTRODUCTION

Blastocystis sp is an anaerobic protist, which commonly colonizes intestines of man and various animals. It is a polymorphic protozoon, which under a variety of conditions can present an array of morphologies, such as vacuolar, multivacuolar, granular, amoeboid and cystic forms (Tan, 2008; Sadaf *et al,* 2012).

Blastocystis sp has been reported in both symptomatic and asymptomatic individuals (El Safadi *et al*, 2014; Ramirez *et al*, 2014; Casero *et al*, 2015). Although asymptomatic infection is very common with *Blastocystis* sp infection, however, a recent study strongly suggests that *Blastocystis* sp is pathogenic (El Safadi *et al*, 2014), causing gastrointestinal disorders, mainly diarrhea, abdominal pain with non-specific gastrointestinal symptoms, such as nausea, vomiting, anorexia, weight loss, flatulence and dizziness (Zaman, 1996). In addition, it has also been reported to cause arthritis and skin disorders including urticaria and palmoplantar pruritus (Valsecchi *et al,* 2003). Increasing numbers of studies in the identification of *Blastocystis* sp have led to a suggestion that it is distributed worldwide and can be considered as an emerging parasitic disease (WHO, 2008).

Blastocystis sp is suggested to be transmitted by a fecal-oral route through consumption of food or water contaminated with human and animal fecal waste (Li *et al,* 2007; Leelayoova *et al,* 2008). Currently, many studies have implicated the zoonotic transmission of this parasite (Duda *et al*, 1998; Yan *et al,* 2007; Souppart *et al,* 2010; Denoeud *et al,* 2011). Seventeen subtypes of *Blastocystis* sp are determined and certain subtypes were reported to infect humans and animals, including subtypes ST1-ST7 (Yan *et al,* 2007).

Blastocystis sp has been isolated from a majority of fecal samples of domestic dogs and cats (Duda *et al*, 1998). Prevalence of *Blastocystis* sp in farm goat was reported as 30.9% (Tan *et al*, 2013). Another study in Malaysia reported 25.2% of chickens are infected with *Blastocystis* sp, with a range of 33.3-100% in different species of free-roaming chickens, whereas no *Blastocystis* sp was observed in caged-chickens (Farah *et al,* 2014).

In Malaysia, there is no reported study on the prevalence and subtypes of *Blastocystis* sp in animals reared by humans in areas endemic for intestinal parasitic infections. Furthermore, there is an absence of study to determine the distribution of *Blastocystis* sp subtypes in animals during dry and wet seasons. We believe that this is the first study to determine the prevalence of *Blastocystis* sp in animals reared by the aborigines in an endemic area of intestinal parasitic infections, during wet and dry seasons.

MATERIALS AND METHODS

Study area and fecal samples collection

The study was conducted in three remote Orang Asli (aborigines) settlements in Temerloh, Pahang, Malaysia, namely, Kampung Penderas village (3.73109°, 102.29112°) located most downstream, Kampung Lubok Wong village (3.76743°, 102.24094°) located in the middle and Kampung Terbol village (3.81391°, 102.23003°) located upstream. The study area is endemic for intestinal parasitic infections (Anuar *et al,* 2012a; 2012b; 2013; Noradilah *et al*, 2017). Fecal collectioms were carried out during the wet (November 2014) and dry (June 2015) season. The wet and dry seasons are determined based on a 2-years total volume of rainfall (mm) recorded in Temerloh

Station (2014 and 2015) obtained from the Malaysian Meteorological Department (MetMalaysia).

The worst floods in Malaysia hit the north eastern parts of the country starting from middle of December 2014 to January 2015 (Baharuddin *et al*, 2015). Being located at the north eastern part of Malaysia, the study areas were heavily flooded during the wet season, which was two months prior to the fecal collection. Fecal collection during the dry season was collected approximately 5 months after the heavy flood.

Animals reared by the Orang Asli people were identified and 127 and 146 fecal samples were collected in the wet and dry season, respectively. The fresh animal fecal samples on the ground were collected using wide-mouth screw-capped labeled containers and placed in individual sealed plastic bags. No additive was added to the fecal samples from domesticated dogs, free-living chickens, goats, ducks, swans, birds and cows. All samples were processed at the Community Laboratory, Department of Parasitology and Medical Entomology, Faculty of Medicine, Universiti Kebangsaan Malaysia Medical Centre, Kuala Lumpur.

The study protocol was approved by the Research and Ethics Committee, Faculty of Medicine, Universiti Kebangsaan Malaysia (FF-2014-219). Permission for animals fecal collection were obtained from the Ministry of Rural and Regional Development Malaysia (reference no. JAKOA/PP.30.032Jld29(04).

Amplification of *Blastocystis* sp DNA

Approximately 200 mg of fecal sample were subjected to DNA extraction using QIAamp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany). Small-subunit (SSU) rDNA of *Blastocystis* sp was PCR amplified using primers BhRDr (5′-GAGCTTTTTAACTGCAACAACG-3′) and RD5 (5′ATCTGGTTGATCCTGCCAGT-3′) (Scicluna *et al*, 2006). Thermocycling was performed as follows: 95°C for 5 minutes; followed by 30 cycles of 95°C for 1 minute, 63.3°C for 90 seconds and 72°C for 1 minute. Amplicons (~600 bp) were separated by 1.5% agarose gel-electrophoresis and subjected to sequencing to determine the subtypes. Sequences were compared with those from GenBank™ using BLAST program (http://www.ncbi.nlm.nih.gov/blast) and were deposited at GenBank™ with accession nos. KX234592 - KX234640 (Table 1)

Statistical analysis

Data obtained during wet and dry seasons were analyzed using descriptive analysis. Proportionate tests (*Z*-test and chi-square) were used to determine the significant difference between the rates of *Blastocystis* infection during the wet and dry seasons.

RESULTS

The detection rate of *Blastocystis* sp in fresh feces from animals during the wet and dry season was 29% (37/127) and 37% (54/146), respectively (Table 2).

Analysis of the partial sequences of *Blastocystis* SSU rDNA showed that the predominant subtype in reared animals during wet season was ST3 (16%) (Table 3), with dog being the most common animal infected (43%), 34% with ST3. Approximately 21% of chickens were infected and the most prevalent subtypes were ST3 and ST7. ST2, ST5 and ST8 were not detected in any of the animals studied.

In the dry season, *Blastocystis* sp ST3 (10%) and ST1 (8%) were the most prevalent subtypes (Table 4). Similar to the wet

Table 1
GenBank accession numbers of the positive *Blastocystis* SSU rDNA sequences detected in the animal fecal samples collected during the wet (November 2014) and dry (June 2015) seasons in Temerloh, Pahang, Malaysia.

Samples code	Source of samples	Subtype	Accession number
Chicken LWA1	Chicken	7	KX234592
Chicken LWA3	Chicken	7	KX234593
Chicken KTA17	Chicken	7	KX234594
Chicken LWA9	Chicken	6	KX234595
Chicken LWAii3	Chicken	9	KX234596
Chicken KTA5	Chicken	3	KX234597
Chicken KTA3	Chicken	1	KX234598
Cat KPA	Cat	1	KX234599
Cat KTA1	Cat	4	KX234600
Goat LWA5	Goat	8	KX234601
Goat LWA6	Goat	4	KX234602
Goat LWA7	Goat	4	KX234603
Goat KPA6	Goat	4	KX234604
Duck KPA2	Duck	1	KX234605
Duck KTA3	Duck	2	KX234606
Duck KTA1	Duck	7	KX234607
Swan KPA1	Swan	3	KX234608
Swan KPA3	Swan	3	KX234609
Dog KPA7	Dog	3	KX234610
Dog KPA4	Dog	3	KX234611
Dog KPA5	Dog	8	KX234612
Dog LWA4	Dog	8	KX234613
Dog KTA1	Dog	1	KX234614
Dog LWA1	Dog	1	KX234615
Dog LWA2	Dog	10	KX234616
Dog KPA14	Dog	4	KX234617
Dog KTA10	Dog	4	KX234618
Chicken KT34	Chicken	7	KX234619
Chicken LW14	Chicken	7	KX234620
Chicken LW21	Chicken	7	KX234621
Chicken KT6	Chicken	6	KX234622
Chicken KT3	Chicken	9	KX234623
Chicken LW6	Chicken	3	KX234624
Chicken LW24	Chicken	3	KX234625
Chicken KP2	Chicken	3	KX234626
Chicken KP1	Chicken	1	KX234627
Dog KP1	Dog	1	KX234628
Dog KT4	Dog	1	KX234629
Duck KP2	Duck	1	KX234630
Dog LW18	Dog	3	KX234631
Dog KP9	Dog	3	KX234632
Dog KT2	Dog	3	KX234633
Dog LW13	Dog	4	KX234634
Dog LW4	Dog	4	KX234635
Duck KP26	Duck	3	KX234636
Goat LW4	Goat	4	KX234637
Goat LW1	Goat	10	KX234638
Bird LW	Bird	10	KX234639
Rat KT	Rat	3	KX234640

Table 2
Prevalence of *Blastocystis* sp in animal feces using PCR-based assay during wet (November 2014) and dry (June 2015) seasons in Temerloh, Pahang, Malaysia.

Animal	Number		Number infected		Prevalence (%)	
	Wet	Dry	Wet	Dry	Wet	Dry
Dog	44	40	19	21	43	52
Chicken	47	57	10	17	21	30
Goat	11	20	3	5	27	25
Duck	11	9	4	4	36	44
Swan	10	10	0	7	0	70
Bird	4	0	1	0	25	0
Cow	0	10	0	0	0	0
Total	127	146	37	54	29	37

season, dog (21/40; 52%) had the highest infection rate, with ST3 the most frequent subtype. *Blastocystis* sp ST7 was the most prevalent subtype detected in chicken (17/57; 30%). As in the wet season ST5 was not detected in any of the animals studied.

No significant difference between the number of *Blastocystis* sp infections was observed during wet and dry seasons (Table 5); however, the number of *Blastocystis* sp ST4 infections in animals is significantly different between wet and dry seasons ($p < 0.05$).

DISCUSSION

In an area where *Blastocystis* sp is prevalent in humans (Anuar *et al,* 2013; Noradilah *et al,* 2017), there is a need to determine the burden of this infection in animals and to postulate the reservoir(s) or source(s) of *Blastocystis* sp infection in the community. In addition, the present study was carried out to determine *Blastocystis* sp infection in animals during wet and dry seasons.

This work presents the first report on the prevalence of *Blastocystis* sp subtypes in animals isolated from an Orang Asli population in an endemic area for intestinal parasitic infections (Anuar *et al,* 2012a; 2012b; 2014). Dogs and chickens roamed freely within the vicinity of their dwellings with feces present around the houses. In the Orang Asli population, dogs were reared to guard the houses and for hunting. The present study reports dogs and chickens were the main animals that harbored *Blastocystis* sp. Coprophagous behavior of dogs and chickens might explain the high prevalence of this infection. The predominance of *Blastocystis* sp infection in dogs might suggest that they are the major source of infection and act as a natural host of this infection in the community. These findings are consistent with a study in Egypt, which reported lesser diversity of *Blastocystis* sp subtypes; 25% of the dogs infected by ST1 and 75% by ST2 (Souppart *et al,* 2010). Higher prevalence of *Blastocystis* sp infection and greater diversity with low proportion of subtypes in dogs were observed during the dry compared to the wet season. The recent flood that inundated the villages five months prior to fecal collection in the

Table 3
Distribution of *Blastocystis* sp subtypes in animals during the wet season (November 2014) in Temerloh, Pahang, Malaysia.

Animal	Number	Number infected									
		ST1	ST2	ST3	ST4	ST5	ST6	ST7	ST8	ST9	ST10
Dog	44	3	0	15	1	0	0	0	0	0	0
Chicken	47	1	0	3	0	0	1	4	0	1	0
Goat	11	0	0	0	1	0	0	0	0	0	2
Duck	11	1	0	3	0	0	0	0	0	0	0
Swan	10	0	0	0	0	0	0	0	0	0	0
Bird	4	0	0	0	0	0	0	0	0	0	1
Total	127	5	0	21	2	0	1	4	0	1	3
Prevalence (%)		4	0	16	2	0	1	3	0	1	2

Table 4
Distribution of *Blastocystis* sp subtypes in animals during dry season (June 2015) in Temerloh, Pahang, Malaysia.

Animal	Number	Number infected									
		ST1	ST2	ST3	ST4	ST5	ST6	ST7	ST8	ST9	ST10
Dog	40	5	0	7	4	0	0	0	4	0	1
Chicken	57	2	0	4	0	0	1	8	0	2	0
Goat	20	0	0	0	4	0	0	0	1	0	0
Duck	9	2	1	0	0	0	0	1	0	0	0
Swan	10	3	0	4	0	0	0	0	0	0	0
Cow	10	0	0	0	0	0	0	0	0	0	0
Total	146	12	1	15	8	0	1	9	5	2	1
Prevalence (%)		8	1	10	5	0	1	6	3	1	1

Table 5
Significance of difference between prevalence of *Blastocystis* sp infection in animals during the wet (November 2014) and dry (June 2015) seasons in Temerloh, Pahang, Malaysia.

Blastocystis sp subtype	Total samples collected		Number infected		Significance of difference	
	Wet	Dry	Wet	Dry	Z-score	*p*-value
All subtypes	131	148	38	56	0.737	0.459
ST1			5	12	1.46	0.144
ST2			0	1	0.935	0.352
ST3			21	15	1.527	0.126
ST4			2	8	2.406	0.016*
ST5			0	0	-	-
ST6			1	1	0.107	0.912
ST7			4	9	1.161	0.246
ST8			0	5	2.105	0.035*
ST9			1	2	0.459	0.646
ST10			3	1	1.179	0.238

*Significant difference at $p < 0.05$.

dry season might be the main accountable factor for the changes in the infection profile as *Blastocystis* sp can be transmitted through contaminated water (Abdulsalam *et al*, 2012; Anuar *et al*, 2013).

Blastocystis sp ST8 was detected in dogs during dry season; hence we suggest that the dogs might contract *Blastocystis* sp ST8 infection through flood water since *Blastocystis* sp ST8 is common in non-human primates and rarely has been reported in non-primate hosts (Stensvold *et al*, 2009). In Colorado, USA wild animals including beaver and muskrats serve as amplification hosts for *Giardia* sp, shedding cysts in their feces and contaminating surface waters downstream from the dams with cyst of *Giardia* in early rainfall (Monzingo and Hibler, 1987). A similar scenario might be happening in our study where feces of non-human primates with *Blastocystis* sp contaminate the downstream area during heavy rainfall and massive flooding. Hence, dogs that wander freely within the study area might have acquired the infection through water run-off from the more upstream area where the habitat of the non-human primates are located.

In Malaysia, studies on the prevalence of *Blastocystis* sp in chickens have been very limited. A study by Farah *et al* (2014) reported an average prevalence of 25.2% in non-caged chickens using *in vitro* cultivation in Jones' medium. This present study provides a more comprehensive set of data on the *Blastocystis* sp subtypes in chickens in Malaysia. During the dry season, more numbers of chickens were detected positive for *Blastocystis* sp, with no additional subtypes detected. As seen in dogs, the distribution of *Blastocystis* subtypes in chickens was diverse with subtype ST7 the most prevalent. Avian *Blastocystis* sp subtypes (ST6 and ST7) are frequently found in Asia (Tan, 2008).

Our findings have added to the statistics of *Blastocystis* sp subtypes in chickens in Malaysia, albeit in aboriginal settlements.

A study on four farms in Malaysia, reported 31% of goats examined positive for *Blastocystis* sp with ST1 predominant, followed by ST7, ST6 and ST3 (Tan *et al*, 2013) comparable with the results of the present study except that ST4 and ST10 were detected in the wet season and ST4 and ST8 in the dry season.

This is the first study in Malaysia of *Blastocystis* sp and serotypes from duck fecal samples. In commercial farms in Japan, 56% of the ducks are infected with *Blastocystis* sp (Abe *et al*, 2002), and Pakandl and Pecka (1992) reported in Czechoslovakia a prevalence of 80%, higher than prevalence observed in the current survey. In addition, *Blastocystis* sp was detected in swan fecal samples but only in the dry season.

Previous studies reported *Blastocystis* sp in cattle (Lee *et al*, 2012; Badparva *et al*, 2015; Zhu *et al*, 2017); however, the current study did not detect *Blastocystis* sp infection in the feces of cattle. This might be due to the low number of samples collected, as these animals were not reared in large numbers in the study community and were always kept in corrals.

It is worth noting: (i) the absence of *Blastocystis* sp ST5 in both wet and dry seasons and that of ST2 and ST8 in the wet season; however ST2 and ST8 was isolated from in duck and dog feces, respectively in the dry season although at low frequency; and (ii) that *Blastocystis* sp ST3 was the most common subtype detected during the wet and dry season, with no statistically significant difference in prevalence.

In summary, the findings in this study show *Blastocystis* sp was prevalent in dogs and chickens reared by Orang Asli people in Temerloh, Pahang. The prevalence of *Blastocystis* sp from animal feces was higher during the dry than the wet season. The occurrence of *Blastocystis* sp ST4 detected in dog and goat feces is significantly higher in the dry than wet season. *Blastocystis* sp isolated showed a diverse range of subtypes, more so during the dry season. Dogs and chickens are postulated as being part of the transmission dynamics of this infection in the Orang Asli community; however, the role of these animals as a potential natural reservoir of *Blastocystis* sp is as yet unclear. Health education pertinent to good hygiene practices, especially safe animal waste handling and disposal should help in reducing the transmission of this infection to this community.

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