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Subtype distribution of *Blastocystis* isolated from humans and associated animals in an indigenous community with poor hygiene in Peninsular Malaysia

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Abstract. *Blastocystis* is one of the most common parasites inhabiting the intestinal tract of human and animals. Currently, human *Blastocystis* isolates are classified into nine subtypes (STs) based on the phylogeny of their small subunit ribosomal RNA (SSU rRNA) gene. Although its pathogenicity remains controversial, the possibility of zoonotic transmission was recognized since eight of the nine STs (except for ST9) have been reported in both humans and animals. A cross-sectional study was conducted to determine the prevalence and subtype distribution of *Blastocystis* isolated from humans and associated animals in an indigenous community with poor hygiene in Malaysia, where the risk of parasitic infection is high. A total of 275 stool samples were collected, subjected to DNA extraction and amplified by PCR assay. The *Blastocystis*-positive amplicons were then purified and sequenced. Phylogenetic tree of positive isolates, reference strains and outgroup were constructed using maximum likelihood method based on Hasegawa-Kishino-Yano+G+I model. The prevalence of *Blastocystis* infection among humans and domestic animals by PCR assay were 18.5% (45/243) and 6.3% (2/32), respectively. Through molecular phylogeny, 47 isolates were separated into five clusters containing isolates from both hosts. Among human isolates, ST3 (53.3%) was the predominant subtype, followed by ST1 (31.1%) and ST2 (15.6%). Chicken and cattle had lower proportions of ST6 (50%) and ST10 (50%), that were barely seen in humans. The distinct distributions of the most important STs among the host animals as well as humans examined demonstrate that there is various host-specific subtypes in the lifecycle of *Blastocystis*.

INTRODUCTION

The genus *Blastocystis* has a worldwide distribution and is classified as an enteric protistan parasite. This parasite which can be isolated from stool samples has the capability of infecting a wide array of vertebrates, including humans (Tan, 2008). Infection of *Blastocystis* is called blastocystosis and it is more prevalent among residents of the developing countries compared to industrialized nations. The factors attributable to the increased risk of infection in such environments consist of poor sanitation, consumption of contaminated food or water and coming into close contact with infected animals (Li *et al.*, 2007; Eroglu *et al.*, 2009; Lee *et al.*, 2012). Although the actual mode of transmission for this infection remains uncertain, the established pathway of infection for this parasite is the fecal-oral route (Yoshikawa

et al., 2004). Apart from the fecal-oral route, *Blastocystis* has also been demonstrated to be zoonotic and waterborne (Leelayoova *et al*., 2008; Parkar *et al*., 2010). The severity of this infection among infected humans remains unassertive as the infected could either be symptomatic or remain asymptomatic (Tan, 2008). Nonetheless, diarrhoea, abdominal pain, anorexia, flatulence, nausea and vomiting are the non-specific gastrointestinal indicators of blastocystosis (Tan *et al*., 2010). However, blastocystosis may also lead to irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) are common chronic gastrointestinal illnesses (Poirier *et al*., 2012; Roberts *et al*., 2014).

In laboratories, culture using Jones medium or Locke medium is the most commonly used method for the diagnosis of *Blastocystis*. This culture method has been considered as a gold standard due to its high sensitivity as compared to direct smear used to detect *Blastocystis* in stool samples (Leelayoova *et al*., 2002; Termmathurapoj *et al*., 2004). Nonetheless, this methodology is laborious and not routinely applied in many laboratories. With the presence of a wide genetic variation between morphologically indistinguishable isolates, molecular approaches for the identification and differentiation of *Blastocystis* have been established (Pandey *et al*., 2015) and are currently acknowledged as the backbone of *Blastocystis* epidemiological research. Polymerase chain reaction (PCR) is a molecular technique, which has been demonstrated with high sensitivity (97%) and specificity (95.9%) towards detecting *Blastocystis* compared to conventional microscopy, in developed and developing countries (Santos & Rivera, 2013).

Following PCR detection, the genetic sequencing and analyses of the small-subunit ribosomal RNA (SSU rRNA) are highly useful in identifying the genetic diversity among *Blastocystis*. Of the 17 described subtypes (ST1-ST17), ST1-ST9 and ST10-ST17 are found in humans and in animals, respectively (Stensvold *et al*., 2007; Santin *et al*., 2011). Among the 9 subtypes found in humans, ST3 is most frequently recognized, followed by ST1, ST2 and ST4, whereas, ST5-ST9 are rare (Stensvold *et al*., 2009; Alfellani *et al*., 2013a). On the other hand, a few of these subtypes were also detected in animals e.g. ST3 in non-human primates, ST5 in cattle and pigs and ST7 in birds (Wang *et al*., 2014). This finding strongly postulates that animals might act as *Blastocystis* reservoirs, which may be interrelated with the potential zoonotic transmission route. Hence, *Blastocystis* subtyping is essential in identifying the sources and potential routes of transmission.

Despite the recent methodological advances in molecular techniques, the molecular epidemiology of *Blastocystis* remains unknown in many parts of the world including Malaysia. More recently, many studies conducted locally have begun to provide more information on the distribution of subtypes among the immunocompromised and symptomatic patients (Tan *et al*., 2013; Ragayan *et al*., 2014). However, data from indigenous community and associated animals are still lacking. Indigenous community is highly vulnerable to parasitic infections due to poor personal hygiene and low quality of life. Moreover, the environmental and cultural circumstances might also increase the chances of them having a close contact with domestic animals, thus, escalating the risk of zoonotic transmission there. Therefore, the present study is aimed to investigate the prevalence and distribution of the isolates of *Blastocystis* subtypes from an indigenous community and associated animals in Pahang, Peninsular Malaysia. Exploring the genetic diversity of *Blastocystis* in distinct hosts is vital to having a better understanding on the epidemiology and dynamics of the transmission of this parasite.

MATERIALS AND METHODS

Ethics statement

The present study was conducted in accordance with the guidelines proposed by the Declaration of Helsinki. All procedures concerning human subjects were endorsed

by the Research Ethics Committee of the Universiti Teknologi MARA, Malaysia (reference number: 600-RMI [5/1/6/]) as the procedures and treatment administered were in conformity with the Ministry of Health, Malaysia. Permission to enter village was also attained from the Ministry of Rural and Regional Development Malaysia (reference number: JAKOA/PP30.052 Jld8) and the district heads of communities. Prior to obtaining the consent of all participants, they were given clear explanation on the objectives and procedures of the study in their local language, Bahasa Melayu. Once the participants have understood the process, written consent was obtained from all the participating adults and from the guardians/parents of the participating children prior to the commencement of the survey. Participants were also well informed that their participation was voluntarily and that they could withdraw from the study at any time without penalties.

Study design and area

A prevalence study was conducted between February and March 2016 in Pahang, Malaysia. Human stool samples were collected from two villages namely Sungai Mas (3°57' N latitude, 103°02' E longitude) and Sungai Jin (3°57' N latitude, 103°01' E longitude) (Figure 1). These villages were located about 42 km away from the town of Kuantan (capital city of Pahang), in a valley region and is considered as a remote area. The tropical climate at the study area records an average temperature ranging between 28°C to 33°C with a mean humidify of 92%. While, the rainfall was measured to have a median of 2650 mm per year. The abundant rainfall and the tropical climate aided in the formation of a thick rainforest vegetation with a few streams in the area of study (Malaysian Meteorological Department, 2016). In addition to the rural location of the study area, majority of the houses were built with bamboo or wood with

Figure 1. Map showing the location of the villages in Peninsular Malaysia involved in the study (stars).

inadequate sanitary facilities and no piped water supply. Hence, the villages were located alongside rivers which act as the main source of water for their daily needs. On the other hand, the source of income for most of the villagers involved farming, rubber tapping as well as collecting and selling forest produce. Based on our observation, most of the domestic animals were allowed to wander freely in the villages.

Collection of human and domestic animal stool samples

Upon carefully disseminating the principles of the study and after acquiring written consents, each participant was provided with a clearly labelled wide mouth screw-cap stool container (with participant's name and identification number) for the collection of stool samples. The participants were also given instructions on how to collect stool samples aseptically. A total of 243 stool samples were collected from the participants. The age of the 243 participants ranged from two to 70 years. During the course of the sample collection, no participants were recorded to experience abdominal pain or diarrhoea (gastrointestinal symptoms). Due to several limitations such as restricted resources and cultural belief against volunteering stool samples, this study relied on a single stool sample collection instead of the ideal three consecutive samplings.

Randomly selected dogs (n=10), ducks (n=2), chickens (n=15), cattle (n=3) and birds (n=2) were the domestic animals of which the fresh stool samples were carefully collected from the environment and the cages. All stool samples were stored in a cool condition throughout the transportation and were then preserved at -20°C prior to subjecting the samples to DNA extraction.

DNA extraction from stool samples

QIAamp® Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) was used to extract DNA from all stool samples, following the manufacturer's protocol. Following extraction, the concentration of the DNA samples was performed using a spectrophotometer (Nanodrop, Thermo Scientific). The extracted DNAs' concentration was then adjusted to 5 µg/mL. The DNA samples were stored at -20° until further use.

DNA amplification by PCR

The SSU rRNA fragments with the size of 550 to 585-base pairs (bp) was amplified from the extracted DNA by conventional PCR using primers F1: 52-GGA GGT AGT GAC AAT AAA TC-32 (Bohm-Gloning *et al.*, 1997) and BHCRseq3: 52-TAA GAC TAC GAG GGT ATC TA-32 (Stensvold *et al.*, 2007). PCR amplification for each sample was performed in a 50 µL reaction volume containing 25 µL TopTaq Master Mix kit (QIAGEN, Hilden, Germany), 17 µL nuclease free water, 4 µL template DNA and 2 µL of each primer under the following conditions: initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s and extension at 72°C for 1 min, followed by a final extension for 10 mins at 72°C. The amplified PCR products were analyzed using 1.5% agarose gel (Vivantis) electrophoresis at 70 V for 110 min (Bio-Rad Laboratories, USA), visualized under ultraviolet light and photographed using Sastec ST GD 1510 (SASTEC™ Instrument Inc., Canada). The screening process was repeated three times to test for reproducibility.

DNA sequencing and phylogenetic reconstruction

The PCR amplified fragments were excised and purified using the GenepHlow™ PCR Cleanup Kit DCF100/DCF 300 (Geneaid Biotech Ltd., Taiwan) following the manufacturer's protocol. The SSU rRNA gene was sequenced using primers F1 and BHCRseq3 using BigDye® Terminator v3.1 Cycle Sequencing Kit (Fisher Scientific, USA). Sequencing was performed using ABI 3730 XL sequencer (Applied Biosystems, Foster City, CA). The SSU rRNA gene sequences obtained in the present study were aligned and manually edited. To infer the genetic similarity, the sequences were tested using BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Referral sequences

of SSU rRNA gene of *Blastocystis* can be obtained from the GenBank Database. Multiple alignments were performed using ClustalX2.1 software and once the sequences were aligned, phylogenetic reconstruction was performed using MEGA 6.06 software (Tamura *et al.*, 2013). The maximum likelihood (ML) phylogenetic tree was reconstructed using the best-fit substitution model, which is the Hasegawa-Kishino-Yano+G (Gamma distribution) + I (Invariant) model with a parametric bootstrap of 1,000 replicates. The SSU rRNA gene sequence of *Proteromonas lacertae* (GenBank accession no. U37108) was used as an outgroup. Sequences generated in this study were submitted to GenBank under accession numbers KX108705-KX108733.

Statistical analysis

Statistical analysis was performed using the SPSS software (Statistical package for the Social Science) for Windows version 24 (SPSS Inc., Chicago, IL, USA). Descriptive analysis was performed with percentage used to explore the positive stool samples and subtype distribution of *Blastocystis*. The associations between each variable (gender and age group) was tested using Pearson's Chi-square (S^2) on proportion, where the difference was considered to be statistically significant with a p value of <0.05.

RESULTS

Prevalence of *Blastocystis* in humans and associated animals by PCR assay

The prevalence of *Blastocystis* infection in humans was 18.5% (45/243) and 6.3% (2/32) in domestic animals as detected by PCR assay. Analysis of the prevalence based on PCR discovered that neither host genders (p=0.979) nor age groups (p=0.284) affected the prevalence significantly. Figure 2 shows the positive PCR results of *Blastocystis*. The PCR products were displayed to be about 550 to 585-bp long.

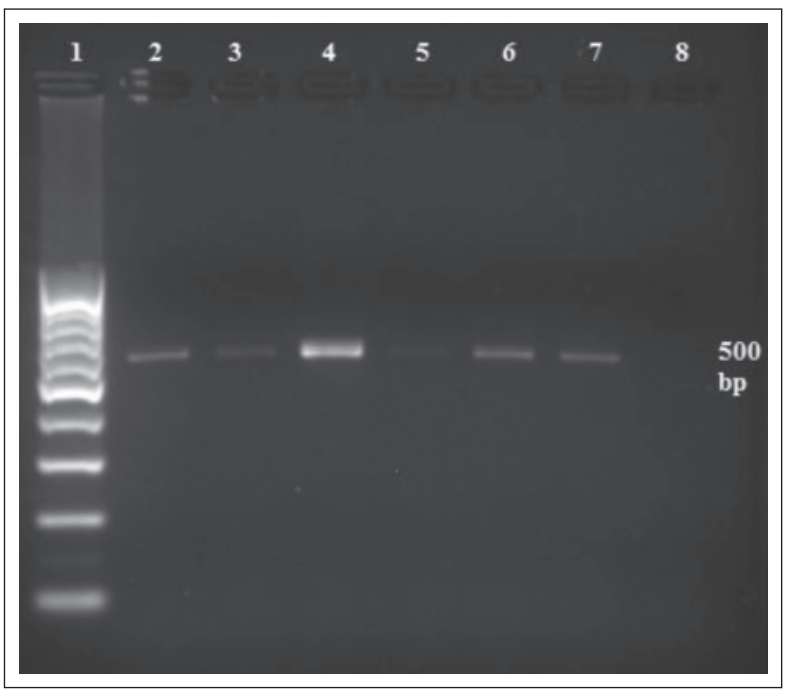

Figure 2. Analysis of PCR products on a 1.5% (w/v) agarose gel. Lane 1, loaded with 100-bp DNA Ladder; Lane 2, positive control; Lane 3-5, *Blastocystis*-positive of humans; Lane 6-7, *Blastocystis*-positive of domestic animals (chicken and cattle); Lane 8, negative control.

Table 1. Distribution of *Blastocystis* subtypes performed on 275 samples comprising isolates from different hosts

	Subtypes (STs)[a]				
	ST1	ST2	ST3	ST6	ST10
Humans (n=45)	14 (31.1%)	7 (15.6%)	24 (53.3%)	0	0
Domestic animals (n=2)	0	0	0	1 (50%)	1 (50%)

n = number of samples positive by PCR assay.
[a] = subtypes detected by PCR and phylogenetic analysis.
ST1 = subtype 1; ST2 = subtype 2; ST3 = subtype 3; ST6 = subtype 6; ST10 = subtype 10.

Characterization of *Blastocystis* subtypes in positive stool samples

As indicated in Table 1, readable sequences that were successfully obtained from humans (45), chicken (1) and cattle (1) hence underwent subtyping analysis. All sequences from different hosts showed 98% to 100% homology to previously published sequences of *Blastocystis*.

Through phylogenetic analysis, 47 isolates were separated into five subtypes, which contained isolates from humans and associated animals in indigenous community. Among human isolates, ST3 (53.3%) was the predominant subtype, followed by ST1 (31.1%) and ST2 (15.6%). Chicken was associated with ST6 (50%) and cattle with ST10 (50%). No mixed subtypes were detected in the representative isolates. Figure 3 depicts phylogenetic relationship between humans, domestic animals and reference sequences based on SSU rRNA gene analysis. Molecular analysis performed on dogs, ducks and birds stool samples were found as negative (data not shown).

DISCUSSION

The transmission of parasitic diseases and the genetic determinants of these infectious diseases can be unravelled through molecular epidemiology (Ramirez *et al*., 2014). Therefore, based on the current findings, the prevalence of *Blastocystis* was estimated at 18.5% in humans and 6.3% in domestic animals, indicating that *Blastocystis* infection was still prevalent and endemic at the surveyed area. The former percentage is in concordance with previous surveys or reports of *Blastocystis* infection in Malaysia, which falls within a prevalence rate of 13.3-24.7% for *Blastocystis* in humans (Abdulsalam *et al*., 2012; Anuar *et al*., 2013). However, the representative percentage calculated for the prevalence of the infection among humans in this study precedes that of the value reported in Thailand and India (Yaicharoen *et al*., 2006; Rayan *et al*., 2010). The high endemicity observed in this study postulates that the indigenous people at the surveyed area were living in an unhygienic condition. A similar pattern was found with other gastrointestinal parasites such as *Entamoeba* and *Giardia* where unhygienic conditions aided in the transmission of these parasites (Anuar *et al*., 2012; Shahrul Anuar *et al*., 2012). Moreover, the spread of the blastocystosis is also made possible with the absence of clean water and a proper sewage system in *Blastocystis* endemic areas. In addition, fecal cross-contamination between infected humans and domestic animals sharing the same water sources seems to be common at this site, as they in close proximity.

It is important to note that the current data was in agreement with Nithyamathi *et al*. (2016), which discovered no difference in the prevalence of the infection between genders, although other studies have stated otherwise. On the contrary, there was also no significant difference in prevalence between age groups in the present findings. A few other studies concurred with our findings in failing to associate age to the prevalence of *Blastocystis*. For instance, no

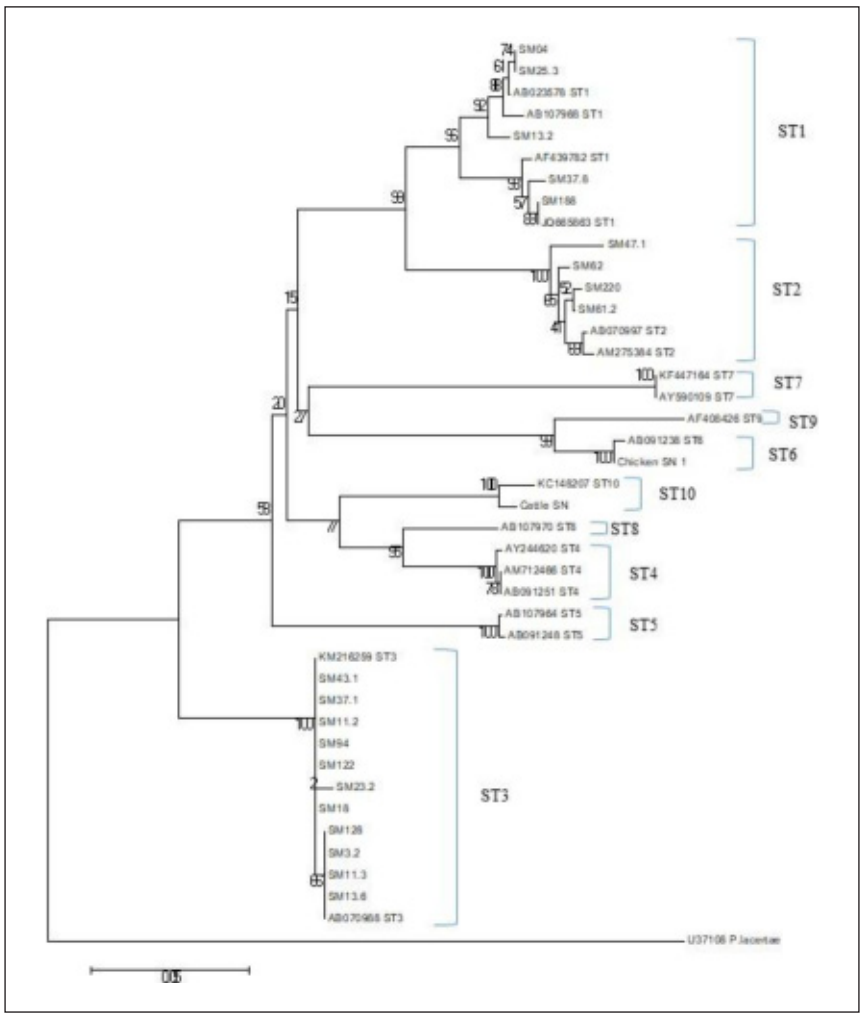

Figure 3. Maximum likelihood tree displaying the relationship among *Blastocystis* isolates, inferred from SSU rRNA sequences. Sequences from GenBank were used for comparison. All human samples were clustered under ST1, ST2 and ST3. Chickens were clustered under ST6 and ST7, while cattle under (ST10).

significant associations were found between the prevalence of *Blastocystis* subtypes and age groups in Libya (Abdulsalam *et al*., 2013). This outcome suggested that the distribution of blastocystosis among host populations showed spatial heterogeneity in regards to the host age and gender. These discrepancies could most likely be attributable to the environmental conditions that affect the efficiency of the fecal-oral route transmission among hosts of varying age and gender (Abdulsalam *et al*., 2013; Abu-Madi *et al*., 2015).

Globally, ST1-ST4 have been identified as the most common subtypes in humans (Yoshikawa *et al*., 2003; Boorom *et al*., 2008; Wong *et al*., 2008; Alfellani *et al*., 2013a). In the present study, ST3 which was identified as a distinct subtype with a wide distribution among the indigenous people is the most prevalent subtype, followed by ST1 and ST2. With the presence of several subtypes, no mixed infections were detected throughout the repeated amplification. Similarly, the absence of mixed infections was also reported in some recent studies carried out

in India and Malaysia (Pandey *et al.*, 2015; Nithyamathi *et al.*, 2016). However, subtype ST1 was found to be predominant in Thailand based on the data demonstrated by Leelayoova *et al.* (2008) and Thathaisong *et al.* (2013). Remarkably, Thathaisong *et al.* (2013) also documented total absence of the ST3 subtype within the community in Thailand. Therefore, the differences in relative abundance of ST1-ST3 suggested that the disparity in transmission efficiency might exist between the subtypes, which include the cyst numbers, cyst survival, exposure to cyst, infectious dose or transmission cycles. Contrary to the discussion above, the incomparable observations could have also been due to differences in PCR protocols between the researchers, study areas and study population, thus, resulting in differential results for subtype distribution. It is also worth noting that ST4 subtype which was absent among human infections in this study, appeared to be prevalent among IBS or *Blastocystis*-positive patients with diarrhoea in Europe (Stensvold *et al.*, 2011; Alfellani *et al.*, 2013b).

In addition, the possibility of human-to-human infection was also observed in the current study as family members from eight different households were identified to be infected with the same subtype of *Blastocystis*. Five households were infected with ST3, two with ST1 and the remaining with ST2. Therefore, sharing poorly maintained household facilities among the family members may have facilitated the human-to-human transmission. This transmission dynamic has been previously demonstrated by Anuar *et al.* (2013), as they discovered that the presence of infected family members with *Blastocystis* sp. to be the primary risk factors for the acquisition of this parasite. Besides, the use of conventional detection methods with insensitive indicators (formalin-ether sedimentation technique and trichrome staining) will fail to properly elucidate the human-to-human transmission route of the parasite (Popruk *et al.*, 2015). However, the successful subtyping of *Blastocystis* as observed in the current study provides solid and conclusive justification about the route of transmission.

As for the domestic animals, the chicken that was examined in the present study was infected with ST6. This subtype is commonly found in avian hosts (Stensvold *et al.*, 2009). In addition, a recent study in Poland stated that domestic chickens may act as natural reservoir for *Blastocystis* ST6 and ST7 (Lewicki *et al.*, 2016). Although ST6 infection seemed to be host specific, the occasional isolation of these subtypes from humans require further epidemiological surveys to explore the host specificity of these subtypes (Yan *et al.*, 2007; Nagel *et al.*, 2012). Secondly, with up to an 80% infection rate, Bovidae or cattle has been indicated as natural hosts for *Blastocystis* ST10 (Cian *et al.*, 2017). Genetic screening further validated that the cattle in this study area was indeed infected with ST10. Furthermore, accumulated study findings reported the predominance of ST10 in cattle across the region including Denmark, USA, Libya, UK and Colombia. This postulated that there was no geographical restriction for this subtype as majority of artiodactyls harboured ST10 (Stensvold *et al.*, 2009; Santin *et al.*, 2011; Fayer *et al.*, 2012; Alfellani *et al.*, 2013a). However, this subtype has never been found infecting humans which is also reflected in the present study, hence, diminishing its role as a potential zoonotic pathogen (Alfellani *et al.*, 2013b).

CONCLUSION

This study reported the presence of STs 1, 2, 3, 6 and 10 and its prevalence among the indigenous community and their domestic animals. Based on the findings of this study, chicken and cattle may be a natural host of *Blastocystis* as well. This was made possible as the epidemiological approach utilized in this study specifically targeted the humans and animals from the same locality and were subjected to a robust molecular technique to further infer the host specificity of this parasite. In conclusion,

our epidemiological approach of targeting humans and animals at the same time in the present study can help to increase our understanding of host-parasite relationships within the selected area and country.

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