IJP: Parasites and Wildlife 11 (2020) 143–148

Contents lists available at ScienceDirect

IJP: Parasites and Wildlife

journal homepage: www.elsevier.com/locate/ijppaw

First subtyping of *Blastocystis* sp. from pet rodents in southwestern China

Yijun Chai[1], Lei Deng[1], Haifeng Liu[1], Jingxin Yao, Zhijun Zhong, Hualin Fu, Liuhong Shen, Ziyao Zhou, Junliang Deng, Yanchun Hu, Guangneng Peng*

The Key Laboratory of Animal Disease and Human Health of Sichuan Province, College of Veterinary Medicine, Sichuan Agricultural University, Chengdu, Sichuan, 611130, China

ARTICLE INFO

Keywords:
Blastocystis sp.
Pet rodent
Subtype
China

ABSTRACT

Blastocystis sp. is a common eukaryotic parasite, which infects humans as well as various other animals. To date, epidemiological data regarding the detection rate and distribution of *Blastocystis* sp. subtypes in pet rodents are lacking in China; the present study aims to fill this gap. A total of 503 fecal samples collected from pets in different locations in southwestern China were screened for the presence of *Blastocystis* sp. using a nested PCR amplification of SSU rRNA method. Forty-two samples (8.35%) tested positive for *Blastocystis* sp. colonization. Two subtypes of *Blastocystis* sp. were identified based on nucleotide sequence homology and phylogenetic analysis: *Blastocystis* ST4 was present in 41 samples, and *Blastocystis* ST17 was found in 1 sample. Our results revealed robust host preference of *Blastocystis* ST4 and confirmed that *Blastocystis* ST17 can also parasitize rodents.

1. Introduction

Blastocystis is a genus containing common single-celled intestinal parasitic protists (Andersen and Stensvold, 2015). *Blastocystis* sp. commonly colonizes the gastrointestinal tracts of humans and a range of other animals (Greige et al., 2018). It is transmitted among hosts through the fecal-oral route (Asghari et al., 2019). Previous studies have shown that *Blastocystis* sp. is associated with irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) (Kumarasamy et al., 2018). However, no study has been able to confirm that *Blastocystis* sp. is the sole etiological agent of either IBS or IBD (Stensvold and Clark, 2016; Shirvani et al., 2019). Therefore, multicenter studies are also required to further investigate the clinical implications of *Blastocystis* sp. with respect to IBS and IBD (Boorom et al., 2008; Wawrzyniak et al., 2013; Rojaleen et al., 2016; Lepczyńska et al., 2017; Aynur et al., 2019). Moreover, studies have indicated that *Blastocystis* sp. is a commensal organism that inhabits the healthy gut, rather than an organism that is only present during gut dysbiosis that is a characteristic feature of metabolic or infectious inflammatory diseases of the lower gastrointestinal tract (Scanlan et al., 2014; Yoshikawa et al., 2016). Several studies have demonstrated the presence of *Blastocystis* sp. in healthy, aymptomatic individuals from Europe, Asia, Africa and South America (Guimaraes and Sogayar, 1993; Moosavi et al., 2012; Pandey et al., 2015; Ben Abda et al., 2017; Nieves-Ramirez et al., 2018). Currently, the pathogenicity of *Blastocystis* sp. remains controversial and unclear (Li et al., 2018; Asghari et al., 2019), which makes it difficult to implement the systematic research approaches commonly used to study other infectious species (Boorom et al., 2008).

Two date, 17 different subtypes of *Blastocystis* sp. have been identified (Cian et al., 2017). Among them, ST1 to ST9 and ST12 have been reported in humans with varying prevalence levels (Ramírez et al., 2016). ST1 to ST8 have been identified in both humans and animals, and considered have zoonotic potential (Song et al., 2017; Xiao et al., 2019). The others strains (ST9 to ST17) have been exclusively identified in either humans or animals; for example, ST9 has only been isolated from humans (Stensvold et al., 2009; Tan, 2008). Studies conducted in different parts of the world show that ST4 is the most common subtype detected in rodents (Katsumata et al., 2018). In addition, ST1 - ST8 (with the exception of ST6), ST13, and ST17 have all been isolated from various rodents (Alfellani et al., 2013a,b; Cian et al., 2017; Katsumata et al., 2018; Valença-Barbosa et al., 2019; Xiao et al., 2019) (Table 2).

In China, over 12 provinces/municipalities have *Blastocystis* sp. infection reported (Wang et al., 2018; Deng et al., 2019). *Blastocystis* sp. has been reported in many animals, such as pigs, cattle, sheep, goats, and cats (Zhu et al., 2017; Wang et al., 2018). However, to date no genetic studies have been conducted on *Blastocystis* sp. isolated from pet rodents in China, and its role as reservoirs of infection for humans and other animals is unknown. *Blastocystis* sp. identified from rodents has

* Corresponding author.
E-mail address: pgn.sicau@163.com (G. Peng).
[1] These authors have contributed equally to this work.

https://doi.org/10.1016/j.ijppaw.2020.01.012
Received 18 September 2019; Received in revised form 24 January 2020; Accepted 25 January 2020

been reported in literature from the USA, France, Singapore, and Japan (Katsumata et al., 2018). Pet rodents are common companion animals that live in close association with the owners, and pet rodents can harbor human pathogens (Jacob et al., 2014). The current study aimed to determine the existence and diversity of *Blastocystis* sp. in rodents being kept as pets in different cities of southwestern China.

2. Materials and methods

2.1. Ethical statement

This study was performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Health, China. Before the initiation of experiments, the protocol of the current study was reviewed and approved by the Institutional Animal Care and Use Committee of the Sichuan Agricultural University under permit. No animals were harmed during the sampling process. Permission was obtained from pet owners or shop managers prior to collection of fecal specimens.

2.2. Study sites

The study was carried out in Sichuan Province, China. This province covers an area of over 486,000 square kilometers, with approximately 83 million people. The sample collection was conducted in four regions of the province (Chengdu, Ziyang, Luzhou, and Dazhou) (Fig. 1).

2.3. Sampling

A total of 503 samples were collected from the following four regions of Sichuan province: Chengdu (n = 311), Luzhou (n = 98), Ziyang (n = 63), and Dazhou (n = 31). The animals sampled were eurasian red squirrel (*Sciurus vulgaris*), eastern chipmunk (*Tamias striatus*), chinchillas (*Chinchilla lanigera*), and guinea pig (*Cavia porcellus*), and Chinese Hamster (*Cricetulus barabensis*) (Table 1). The fecal samples were collected between September 2018 and May 2019 in Sichuan Province, China. Each rodent was kept in a separate cage. Approximately 200 mg of fresh fecal samples were collected using sterile gloves from the excrement disc at the bottom of the cage immediately after defecation. Samples were then transferred to sterile plastic containers marked with the species and sampling date. The fecal samples were transported to the laboratory by storing along with ice packs within 24 h of collection. All study animals were examined, and no pronounced clinical signs were apparent during sampling.

2.4. DNA extraction

Genomic DNA was extracted directly from fecal samples

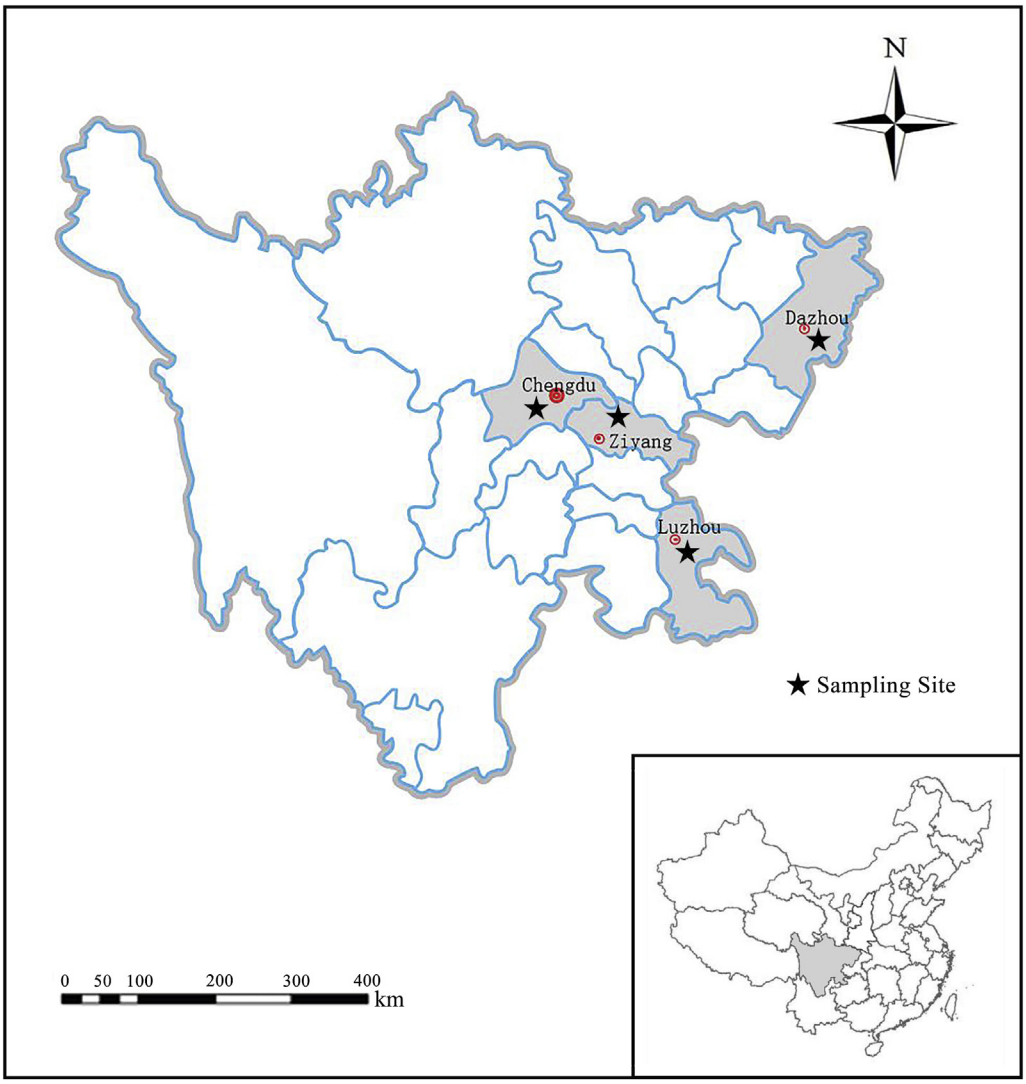

Fig. 1. Sampling sites in Sichuan Province of China.

Table 1
Detection rate and subtypes of *Blastocystis* sp in rodents from different sources in Southwestern China.

Location	Host	Scientific name	No. of examined	No. of positive	Detection rate (%)	Species (n)
Chengdu	Eurasian Red Squirrel	*Sciurus vulgaris*	72	7	9.72	ST4 (7)
	Eastern Chipmunk	*Tamias striatus*	108	3	2.78	ST4 (3)
	Chinchilla	*Chinchilla lanigera*	72	3	4.17	ST4 (2), ST17 (1)
	Guinea pig	*Cavia porcellus*	59	10	16.95	ST4 (10)
Subtotal			311	23	7.40	ST4 (22), ST17 (1)
Luzhou	Chinese Striped Hamster	*Cricetulus barabensis*	98	12	12.24	ST4 (12)
Ziyang	Eastern Chipmunk	*Tamias striatus*	63	5	7.94	ST4 (5)
Dazhou	Guinea pig	*Cavia porcellus*	31	2	6.45	ST4 (2)
Total			503	42	8.35	ST4 (41), ST17 (1)

Fig. 2. Phylogenetic relationships among nucleotide sequences of *Blastocystis* partial small subunit ribosomal RNA (SSU rRNA) genes. The neighbor-joining method was used to construct the trees from the Kimura-2-parameter model. Branch numbers represent percent bootstrapping values from 1000 replicates, with values of more than 50% shown in the tree. Each sequence is identified by its accession number, subtypes, host origin, and country. *Blastocystis* subtypes identified in the present study are indicated in bold-type.▲ are subtypes in this study.

(approximately 200 mg) using the QIAamp DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany), in accordance to the procedures recommended by the manufacturer. The extracted DNA was stored at −20 °C until PCR analysis.

2.5. Subtyping of Blastocystis sp.

All DNA preparations were screened for the presence of *Blastocystis* sp. by PCR amplification of the barcode region (a fragment of ~510 bp) of the SSU rRNA gene. The primers and cycling parameters were in accordance to those described by Scicluna et al. (2006). TaKaRa Taq DNA polymerase (TaKaRa Bio Inc., Tokyo, Japan) was used for all of the PCR reactions. A negative control with no DNA added was included in all of the PCR tests. PCR products were subjected to electrophoresis in a 1.5% agarose gel and visualized by staining the gel with ethidium bromide.

2.6. Sequence analysis

All positive PCR products were directly sequenced on an ABI PRISMTM 3730 DNA Analyzer (Applied Biosystems, Foster, CA, USA), using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). Nucleotide sequences obtained in the present study were subjected to BLAST (http://www.ncbi.nlm.nih.gov/blast/)., aligned with each other, and analyzed. Reference sequences were downloaded from the GenBank (http://www.ncbi.nlm.nih.gov). The sequences were aligned using Clustal X 2.0 (http://www.clustal.org/) to determine the *Blastocystis* sp. subtype. The nucleotide sequences generated in the present study have been deposited in GenBank (Table S1).

2.7. Phylogenetic analyses

A neighbor-joining tree was constructed to assess the genetic relationship among the *Blastocystis* subtypes obtained in the present study and those identified in previous studies using the software Mega 7 (http://www.megasoftware.net/). The sequences of the barcode region of *Blastocystis* sp. were trimmed using trimAl (Capella-Gutiérrez et al., 2009). Evolutionary distances were calculated using the Kimura two-parameter model. The reliability of the trees was assessed by bootstrap analysis with 1000 replicates.

Table 2
Subtypes and positive samples of *Blastocystis* sp. detected from rodents in the world.

Country	Host (scientific name)	Technique	Number of samples	Number of Positive	Prevalence (%)	Subtypes(n)	References
Brazil	Cursorial akodont (*Akodon cursor*)	PCR	1	1	100		Valença-Barbosa et al. (2019)
	House Rat (*Rattus rattus*)	PCR	1	1	100	ST3 (1)	
	Montane Grass Mouse (*Akodon montensis*)	PCR	2	2	100		
	Brazilian forest rodent (*Atlantic Forest Nectomys*)	PCR	1	1	100		
	Brazilian forest rodent (*Atlantic Forest Nectomys*)	PCR	2	2	100	ST8 (1)	
Japan	Brown rat (*Rattus norvegicus*)	PCR				ST4 (11)	Katsumata et al. (2018)
	Rat (*Vole*), Guinea pig (*Cavia porcellus*) Unclear specific host	PCR				ST7 (2)	
Indonesia	Polynesian rat (*Rattus exulans*)	PCR	12	?		ST4 (12)	
	Polynesian rat (*Rattus exulans*)	PCR	77	10	13	ST4 (9)	Yoshikawa et al. (2016)
France	Norway rat *(Rattus norvegicus)*	qPCR	2	1	50	ST4	Cian et al. (2017)
	Capybara *(Hydrochoerus hydrochaeris)*	qPCR	5	3	60	ST2 (1),ST5 (1)	
USA	Rat (*Rattus* sp.)	qPCR	5	5	100	ST4 (5)	Noël et al. (2003)
	Guinea pig (*Cavia porcellus*)	qPCR	2	2	100	ST4 (2)	Noël et al. (2005) Yoshikawa et al. (1998) Leipe et al. (1996)
UK	Bank vole *(Clethrionomys glareolus)*	Sequencing	32	1	3.13	ST5	Alfellani et al. (2013a,b)
	Wood mouse *(Apodemus sylvaticus)*	Sequencing	13	1	7.69	ST3	
Belgium Croatia	Chinchilla *(Chinchilla lanigera)*	Sequencing	5	2	40	ST3 (2)	
Poland	Yellow necked mouse (*Apodemus flavicollis)*	Sequencing	1	1	100	ST3	
Libya	Gundi (*Ctenodactylus gundi*)	Sequencing	4	1	25	ST17	
Colombia	House Rat (*Rattus rattus*)	STs	3		无	ST2 (3)	Ramírez et al. (2014)
China	Brown rat (*Rattus norvegicus*)	PCR	108	4	3.7	ST4 (4)	Deng et al. (2019)
China	Trogopterus xanthipes (*Rodentia*)	PCR	69	21	30.4	ST1 (8),ST3 (4),ST13 (9)	Xiao et al. (2019)
China	Eurasian Red Squirrel (*Sciurus vulgaris*)	PCR	72	7	9.72	ST4 (7)	This study
	Eastern Chipmunk (*Tamias striatus*)	PCR	171	8	4.68	ST4 (8)	
	Chinchilla (*Chinchilla lanigera*)	PCR	72	3	4.17	ST4 (2),ST17 (1)	
	Guinea Pig (*Cavia porcellus*)	PCR	90	12	13.33	ST4 (12)	
	Chinese Striped Hamster (*Cricetulus barabensis*)	PCR	98	12	12.24	ST4 (12)	
	Eurasian Red Squirrel (*Sciurus vulgaris*)	PCR	72	7	9.72	ST4 (7)	
	Eastern Chipmunk (*Tamias striatus*)	PCR	171	8	4.68	ST4 (8)	

2.8. Statistical analysis

Statistical analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). A chi-squared test was used to compare the occurrence of *Blastocystis* sp. in different pet markets and different species. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Detection rate of Blastocystis sp. in pet rodents

The overall minimum prevalence of *Blastocystis* sp. in pet rodents was 8.35% (42/503; 95% CI: 0.06–0.11). The detection rate in different rodent species ranged from 4.17 to 13.33% (Table 1), and differed significantly between the five pet species ($\chi^2 = 9.699$, $df = 4$, $p < 0.05$). There was no significant difference in the prevalence of *Blastocystis* sp. between the four locations tested, which ranged from 6.45 to 12.24% ($\chi^2 = 2.473$, $df = 3$, $p > 0.05$).

3.2. Distribution of Blastocystis sp. subtypes in pet rodents

Two *Blastocystis* sp. subtypes were successfully sequenced, and all SSU rRNA –positive PCR samples were sequenced and phylogenetically analyzed (Fig. 2). Two subtypes (ST4 and ST17) were identified based on the phylogenetic tree (ST4, ST17), both of which are known to have zoonotic potential (Table 1, Fig. 2). ST4 was the predominant subtype, and was widely distributed among the different species of rodents and locations (Table 1). Additionally, ST17 was discovered in a *Chinchilla*, which is a newly identified host for this subtype.

3.3. Phylogenetic analyses

The sequences identified in this study were aligned to known sequences downloaded from GenBank. Thirty-six sequences clustered with subtype ST4A (Genebank accession number MH127500 form *Rattus norvegicus* in Japan). Five sequences clustered with ST4B, which has been identified in human in Germany and Japan (AY244619, AY244621). However, only one sequence clustered with ST17, which has been identified in the *North African gundi* in the UK (Genebank accession number KC148208) (Fig. 2).

4. Discussion

Blastocystis sp. is the most frequent parasite colonizing in humans and a variety of animals (Meloni et al., 2011; Yoshikawa et al., 2012). Some studies have found that infection with *Blastocystis* sp is linked to gastrointestinal and nutritional disorders in both developing and developed countries (Seguí et al., 2018). However, other studies have shown that the presence of *Blastocystis* may be an indicator of good intestinal health (Andersen and Stensvold, 2015).

Our results revealed a *Blastocystis* sp. prevalence of 8.35% in non-diarrheal pet rodents, which is lower than that found in wild rodents in an Indonesian community (13%) (Yoshikawa et al., 2016). The

difference is likely due to the studying animals were wild rodents in Indonesia and pet rodents in China. Generally, shopkeepers in this study cleaned rodent cages regularly, provided clean water, used chlorine for disinfection, and have good sanitary conditions, which may explain the low prevalence of *Blastocystis* sp. in this study.

Previous studies have shown the global prevalence of ST4 in rodents. This subtype predominates in rodents such as brown rats in China, Indonesia, the Philippines, and Japan, and guinea pigs in the UK (Leipe et al., 1996; Abe, 2004; Belleza et al., 2016; Katsumata et al., 2018; Wang et al., 2018). Four subtypes, ST1–ST4 have the highest prevalence (more than 90%) in humans (Cian et al., 2017). Recent studies have revealed that ST4 is the common subtype in Europe, but is rare in other countries (Forsell et al., 2016, 2017; Deng et al., 2019; Gong and Liu, 2019). A few studies have examined the prevalence of ST4 in humans in the Zhejiang and Yunan provinces of China (Deng et al., 2019). Another study in China detected only three known subtypes (ST1, ST3, and ST13) in flying squirrels (Xiao et al., 2019). Previous studies have shown that ST4 has a peculiar geographical distribution and that ST4 is a subtype of *Blastocystis* that is most influenced by geography and lifestyle (Beghini et al., 2017; Forsell et al., 2017). Further studies are required to investigate the mode of transmission of the *Blastocystis* ST4 subtype in China. Unlike other *Blastocystis* subtypes that are commonly found in humans, rodents appear to constitute the main animal reservoir of ST4 (Stensvold et al., 2009). In China, *Blastocystis* sp. may be transmitted by contaminated water to humans indicating that ST4 is more likely to spread between humans and animals (Deng et al., 2019) (Table 2).

To our knowledge, this was the first study on *Blastocystis* sp. subtypes in pet rodent hosts in China. ST4 was the most common subtype of *Blastocystis* sp. in the rodents studied. Additionally, this was the first study to subtype *Blastocystis* sp. from *chinchillas*; none of the previous studies have reported the presence of *Blastocystis* sp. ST17 in China. ST17 was only detected in only one chinchilla from a pet store in Chengdu; however, the existence of this subtype should be further studied by examining additional samples from this and other geographical origins (AbuOdeh et al., 2019; Martinez-Hernandez et al., 2020). The role of pet rodents in transmitting ST4 and ST17 subtypes should be further evaluated. Our findings suggest that pet rodents may act as potential reservoirs for zoonotic *Blastocystis* sp. Further studies are needed to determine the distribution of *Blastocystis* subtypes in the pet and human populations in this region.

Declaration of competing interest

There is no conflict of interests.

Acknowledgments

The study was financially supported by the National Science and Technology Department “13th five-year” Special Subproject of China (No.2016YFD0501009) and Chengdu Giant Panda Breeding Research Foundation (CPF2017-12, CPF2015-09). The funders contributed to the study design and data collection.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2020.01.012.

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