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Investigation and genotypic study on the infection of wild mouse *Blastocystis* sp. in Guangdong and Chongqing

[Investigação e estudo genotípico sobre a infecção de camundongos selvagens Blastocystis sp. em Guangdong e Chongqing]

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ABSTRACT

Blastocystis are common digestive tract parasites in humans and animals, extensively parasitic in humans and other primates. They exhibit extensive genetic diversity; Currently, 17 subtypes (STs) and some populations called non mammalian and avian STs (NMASTs) have been proposed. To understand the infection status and genotype distribution of *Blastocystis sp*. in wild mouse, this study used PCR technology to study the fecal DNA samples of 111 *Leopoldomys edwardsi* and 117 *Berylmys bowersi* collected from Guangdong and Chongqing. Among 228 fecal samples, 4 samples were positive for *Blastocystis* sp., with a total infection rate of 1.8% (4/228). Four positive samples formed two subtypes of ST3 and ST4, all of which were zoonotic genotypes. This article aims to investigate the infection status and genotype distribution of wild mouse *Blastocystis* sp., which will help reduce the infection of this pathogen to animals and thereby reduce their risk of zoonotic transmission.

Keywords: Subtypes, wild mouse, *Blastocystis* sp.

RESUMO

Blastocystis são parasitas comuns do trato digestivo em humanos e animais, amplamente parasitados em humanos e outros primatas. Atualmente, foram propostos 17 subtipos (STs) e algumas populações denominadas STs não mamíferos e aviários (NMASTs). Para entender o status da infecção e a distribuição do genótipo de Blastocystis sp. em camundongos selvagens, este estudo usou a tecnologia PCR para estudar as amostras de DNA fecal de 111 Leopoldomys edwardsi e 117 Berylmys bowersi coletadas em Guangdong e Chongqing. Entre 228 amostras fecais, 4 amostras foram positivas para Blastocystis sp. com uma taxa de infecção total de 1,8% (4/228). Quatro amostras positivas formaram dois subtipos de ST3 e ST4, todos os quais eram genótipos zoonóticos. Este artigo tem como objetivo investigar o status da infecção e a distribuição de genótipos de Blastocystis sp. em camundongos selvagens, o que ajudará a reduzir a infecção desse patógeno em animais e, assim, reduzir o risco de transmissão zoonótica.

Palavras-chave: subtipos, camundongo selvagem, Blastocystis *sp*

INTRODUCTION

Blastocystis sp. is a relatively common digestive tract parasite in humans and animals. It is widely parasitic in humans and other primates, such as dogs, pigs, cats, mice, rats, rabbits, guinea pigs and poultry (Yamada *et al*., 1987; Teow *et al*., 1992; Quílez *et al*., 1995; Abe *et al*., 2002; Stensvold *et al*., 2009; Asghari *et al*., 2019). This microorganism is perchance the most widespread human intestinal parasite globally, with projected one billion contagions in the world (Andersen and Stensvold 2016; Seguí *et al*., 2018; Paulos *et al*., 2018). Its scientific signs include irritable bowel syndrome (IBS) like symptoms, diarrhea, abdominal pain, nausea, vomiting, anorexi andcramps (Kaya *et al*., 2007; Shariati *et al*., 2019). However, its role in pathogenicity and IBS is still indistinct and controversial until now (Shariati *et al*., 2019).

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In previous studies, wildlife was a foremost cause of zoonotic diseases (Jones *et al*., 2008). Rodents are usual reservoirs and vectors of pathogens, and wild rodents play a significant role in the transmission of zoonotic parasitic protozoa. At present, with the incessant expansion of human outdoor activities, the probability of direct and indirect interaction between humans and wild rats is also increasing. Therefore, it is of great public health consequence to investigate the infection and genotype of *Blastocystis* carried by wild mice.

Due to the round or oval shape of *Blastocystis* sp., they are often confused with other pathogenic organisms when viewed under a microscope (Boreham and Stenzel, 1993), and morphological observations cannot be used to identify and distinguish the genetic differences between subtypes, therefore molecular bioassays are gradually replacing these methods (Wawrzyniak *et al*., 2013; Ismail *et al*., 2013; Not *et al*., 2020). Studies have shown that using a conventional Polymerase Chain Reaction, Quantitative real time polymerase chain reaction, quantitative real time polymerase chain reaction, rt-qPCR is highly subtle and proficient for identification and typing of *Blastocystis* sp. based on partly or fully amplified SSU-rDNA genes (Wawrzyniak *et al*., 2013; Stensvold and Clark 2016). SSU r RNA gene is highly preserved and has no significant difference between species. It can be used as a marker of gene sequence of different subtypes of *Blastocystis* sp., and can be successfully used for detection, population development analysis and subtype analysis of *Blastocystis* sp. (Villegas-Gómez *et al*., 2016; Asghari *et al*., 2021). Therefore, this study augmented the SSU rRNA gene sequence of *Blastocystis* using PCR technology to investigate and examine the infection status of *Blastocystis* in wild rats in Guangdong and Chongqing, China, providing new research data for the host and geographical distribution of *Blastocystis* infection.

MATERIALS AND METHODS

From November 2017 to January 2018, 111 and 117 dead wild rats were purchased from markets in Chongqing and Guangdong provinces, China, respectively. A total of 228 experimental animal samples were transported to the laboratory. After PCR sequencing of the *cox1* gene in the mitochondria of the tested samples, species identification was conducted: the mt-cox1 sequences of these two wild rats were 99% homologous with the previously reported Chinese mouse sequences (GenBank registration number: KM434322.1) and the previously published sequences from Vietnam (JN105105.1), respectively. They were identified as *Leopoldomys edwardsi* and *Berylmys bowersi*. Number the experimental samples, record their gender and origin, and store them at -20℃ for future use.

Remove, thaw, and place the frozen wild mice in a biosafety cabinet 6 hours in advance. Using forceps, scissors, and other test instruments that have been high-temperature disinfected, cut along the midline of the abdomen of the wild rat, expose the abdominal cavity, and take all fecal samples from the anus to the small intestine in a 1.5ml EP tube. Clean and disinfect each test instrument taken. Mark the extracted rat fecal sample tube and store it at -20℃ for future use.

Refer to E.Z-N.A ® Extract DNA from mouse fecal samples using the instructions of the fecal DNA kit**.**

Molecular identification was performed by amplifying a region of the SSU rRNA using previously reported primers (Clark, 1997; Scicluna *et al*., 2006). Briefly, the method includes a usual PCR with the primers RD5-R1 (ATCTGGTTGATCCTGCCAGT) and BhRDr-F1 (GAGCTTTTTAACTGCAACAACG) amplifying and 600 base pair (bp) fragment of the small subunit ribosomal RNA gene (SSU rRNA) (TABLE 1). The PCR reaction system consisted of 25μL: 15.8uL ddH_2O, 2.5uL 10×PCRbuffer, 0.25μL upstream primer, 0.25μL downstream primer, 2uL DNTPs, 1.5uL MgCl2, 0.2uL ExTaq and 2.5μL genomic DNA (TABLE 2). Samples in the C1000 Touch^{TM} Thermal Cycler (BioRad, USA) under the following conditions: 94℃ for 5 min, then 94℃ for 45s, 58℃ for 45s (annealing) and 72℃ for 1 min for 35 cycles, with a final extension at 72℃ for 7 min.

Table 1. Primers for PCR of *Blastocystis* sp.

Site	Sequence (5'→3')
BhRDr-F1	GAGCTTTTTAACTGCAACAACG
RD5-R1	ATCTGGTTGATCCTGCCAGT

Table 2. The PCR Reaction System of *Blastocystis* sp.

Reagent	Volume（μL）
ddH_2O	15.8
10×PCRbuffer（Mg2+ free）	2.5
DNTPs	2
MgCl2	1.5
F	0.25
R	0.25
ExTaq	0.2
DNA	2.5
Total	25

After the reaction, the product was detected by electrophoresis, and if bright bands appeared at the length of the target fragment, the product was sent to Shanghai Shengong Bioengineering Company for bidirectional sequencing.

The afresh generated sequences in the present study and previously published SSU rRNA sequences of *Blastocystis* sp. were allied using the software MAFFT 7.122. using *Blastocystis pythoni* (GenBank accession number MT302174) as an outgroup (Yoshikawa *et al.*, 2003). The concatenated nucleotide sequences of SSU rDNA. The aligned sequences were then concatenated to form a single contig. The poor blocks were excepted from the contig using Gblocks 0.91b (http://phylogeny.lirmm.fr/phylo_cgi/one_task.cgi?task_type=gblocks) using default parameters (Talavera and Castresana 2007). Phylogenetic analyses were conducted using two methods: Bayesian inference (BI) and Maximum likelihood (ML). BI analysis was run in MrBayes 3.1.1 as described previously (Yoshikawa *et al.*, 2003; Ronquist and Huelsenbeck, 2003). Phylograms were drawn using the program FigTree v.1.4.

The four wild rat *Blastocystis* sp. sequences obtained in this experiment were stored in the GenBank database according to the following login numbers: login numbers MT302172 – MT302175.

RESULTS AND DISCUSSION

The sequencing results showed that through ordinary PCR amplification of 228 fecal DNA samples from wild rats in Guangdong and Chongqing, China, 4 samples were positive for *Blastocystis*, with a total infection rate of 1.8% (4/228), of which 2 were positive from Guangdong region and 2 were positive from Chongqing region (Table 3). The results of this study were significantly lower than the infection rate of 19.2% of wild rats in a certain area of southwest Iran (Seifollahi *et al.*, 2016), lower than the infection rate of 13.0% of *Blastocystis* sp. mouse from Indonesian communities with poor sanitary conditions (Yoshikawa *et al.*, 2016), and significantly lower than the infection rate of 37.5% of rodents in the United Arab Emirates (AbuOdeh *et al.*, 2019). There was no significant difference in infection rate between the two regions and the species of wild mice. The reasons for this result may be related to many factors such as animal source area, animal species, sampling season, sample quantity and detection method.

Table 3. The prevalence of *Blastocystis* sp. infection in wild rats in Guangdong province and Chongqing Municipality,China

Geographical locations	Species	Sample size	Positive number	Prevalence (%)
Guangdong	*Berylmys bowersi*	117	2	1.71
Chongqing	*Leopoldomys edwardsi*	111	2	1.80
Total		228	4	1.80

In the tree, 3 positive strains had completely identical sequences and 100% homology with the Thai Bacillus thuringiensis isolate (Genbank: MH197686), with a genotype of ST4; One positive strain has 100% homology with an Indian Bacillus thuringiensis isolate (Genbank: MK719686), with a genotype of ST3 (Fig. 1).

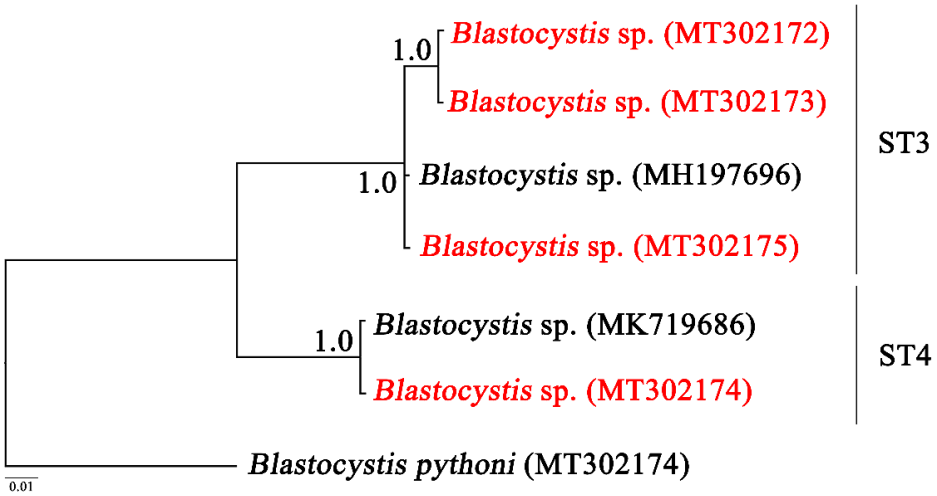

Figure 1. Inferred phylogenetic relationship among species from *Blastocystis* sp. The concatenated amino acid sequences of SSU rDNA were analyzed utilizing Bayesian analysis (BI), using *Blastocystis python* as an outgroup.

At present, there is evidence that ST1-ST4 genotypes in 9 species of *Blastocystis* sp. ST(ST1-ST9) that can be infected by humans account for more than 90% of all cases. Studies have shown that ST3 is the most prevalent genotype in all countries and is also the genotype with the largest number detected in infected individuals. According to domestic and foreign *Blastocystis* sp. surveys, ST3 is the main genotype infected with *Blastocystis* sp. hominis, and the infection rate ranges from 44.1% to 64.9% (Alfellani *et al*., 2013). ST4 is the second most common genotype of *Blastocystis* sp. hominis infection in the UK and is also common in Europe. In a Danish study (Stensvold *et al*., 2011), more than 70% of the strains of *Blastocystis* sp. identified in patients with acute diarrhea were ST4. However, most studies still show that ST4 is mainly hosted by rodents (Noël *et al*., 2005; Katsumata *et al*., 2018), and this study also proves this conclusion.

The research on *Blastocystis* sp. disease in China started late, and mostly focused on the report of human *Blastocystis* sp., and the report on *Blastocystis* sp. infection in wild mice was less. The two *Blastocystis* sp. genotypes identified in this study are closely related to human *Blastocystis* sp. infection, suggesting that wild mice may be the source of human ST3 and ST4 infection and have the potential of zoonosis. Although the sample size is small, it also provides a way to consider that wild rats can serve as important hosts for *Blastocystis* sp. zoonotic genotypes. Therefore, expanding the sample size and further study on *Blastocystis* sp. infection in representative wild rats in different regions and species can provide a more scientific reference for the prevention and control of *Blastocystis* humanis.

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