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Prevalence and molecular subtyping of *Blastocystis* sp. in rabbits in Henan, Central China

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Abstract: Species of *Blastocystis* Alexieff, 1911 are anaerobic intestinal protists found in humans and many kinds of animals that mainly cause diarrhea, abdominal pain and other clinical symptoms. At present, data on the prevalence and subtype diversity of species of *Blastocystis* in domestic rabbits are very limited. The purpose of this study was to characterise the infection rate and gene subtype distribution of *Blastocystis* sp. in domestic rabbits in Henan Province, Central China, and provide foundation for prevention and control of the disease caused by *Blastocystis* sp. in domestic rabbits. DNA was extracted from 286 fresh rabbit faecal samples collected from four areas of Henan Province, Central China. All DNA samples were screened using PCR and positive samples were sequenced to identify individual subtypes based on the small ribosomal subunit (SSU rRNA) gene. The overall infection rate of *Blastocystis* sp. in domestic rabbits in Henan Province was 15% (43/286). Three subtypes were identified, including ST1 (26/43, 60%), ST3 (5/43, 12%) and ST7 (12/43, 28%), all of which belonged to potentially zoonotic subtypes, ST1 was the dominant gene subtype. These results showed that infection with *Blastocystis* sp. was common in domestic rabbits in Henan Province, Central China, and was represented by zoonotic subtypes. Therefore, special attention should be paid to reduce the risk of transmission of *Blastocystis* sp. from domestic rabbits to humans.

Keywords: *Blastocystis* sp., SSU rRNA, epidemiological characteristics, gene subtype, rabbits

Species of *Blastocystis* Alexieff, 1911 are anaerobic intestinal parasites widely distributed all over the world (Cian et al. 2017). They can infect humans and many other animals (Tan 2004, 2008, Skotarczak 2018). The main transmission route is faecal-oral transmission (Yoshikawa et al. 2004). At present, there is still a lot of controversy about the pathogenicity of *Blastocystis* spp. in humans. Although many scholars believe that it is a pathogen (Carrascosa et al. 1996, Levy et al. 1996, Leelayoova et al. 2004, Andiran et al. 2006, Roberts et al. 2014), others still doubt the role of *Blastocystis* sp. in human diseases (Tungtrongchitr et al. 2004, Leder et al. 2005). Studies have shown that ingestion of food or water contaminated by *Blastocystis* sp. can cause infection (Ithoi et al. 2011, Lee et al. 2012a).

Infection with species of *Blastocystis* sp. is characterised by asymptomatic or mild abdominal pain, diarrhea and chronic urticaria (Nagel et al. 2012, Légeret et al. 2020). A recent study reported that *Blastocystis* sp. can cause acute gastroenteritis (Bhat Yellanthoor 2020), which may be related to the difference in human immune status or the pathogenicity of different subtypes of *Blastocystis* sp.

Blastocystis sp. has high morphological and genetic diversity. Based on the study of its small ribosomal subunit (SSU rRNA) gene loci, 28 subtypes (STs) have been identified to date (Maloney et al. 2019, 2020, 2021, Kaczmarek et al. 2020, Maloney and Santin 2021). Twelve subtypes, including ST1-ST10, ST12 and ST14 have been found in humans (Khaled et al. 2020, Ma et al. 2020).

Among these 12 subtypes, all are also found in other mammals and birds except ST9 (Hublin et al. 2021). The same subtype of *Blastocystis* sp. has also been found in patients with *Blastocystis* sp. and animals in close contact with them. A pet breeder in Poland and his pet dog were infected with *Blastocystis* subtype ST7 (Kaczmarek et al. 2020).

In Australian zoos, keepers and five species of primates and a wombat were infected with *Blastocystis* subtypes ST1

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Table 1. Prevalence and subtype distribution of *Blastocystis* in various rabbits in Henan, Central China.

Factors		Common name	No. tested	No. positive	Prevalence %	Subtypes (n)
Shangqiu	Farm 1	Chinese black rabbit	2	2	100	ST1 (1), ST3 (1)
	Age (years)	≤1	0	0	0	
		1~2	1	1	100	ST1 (1)
		≥2	1	1	100	ST3 (1)
		New Zealand white rabbit	14	6	43	ST1 (2), ST3 (4)
	Age (years)	≤1	11	3	27	ST1 (2), ST3 (1)
		1~2	0	0	0	
		≥2	3	3	100	ST3 (3)
Zhumadian	Farmhouse 1	New Zealand white rabbit	15	1	7	ST1 (1)
	Age (years)	≤1	15	1	7	ST1 (1)
		1~2	0	0	0	
		≥2	0	0	0	
	Pet trading market	Belgian hare	24	0	0	
	Age (years)*	≤1	15	0	0	
		1~2				
		≥2				
		Bluish blue rabbits	1	0	0	
	Age (years)	≤1	1	0	0	
		1~2				
		≥2				
		Dutch-belted rabbit	16	0	0	
	Age (years)*	≤1	12	0	0	
		1~2				
		≥2				
		Harbin white rabbit	2	0	0	
	Age (years)	≤1	1	0	0	
		1~2	1	0	0	
		≥2				
		New Zealand white rabbit	17	0	0	
	Age (years)*	≤1	13	0	0	
		1~2				
		≥2				
Anyang[a]	Farm 2	Chinese black rabbit	2	0	0	
	Age (years)	≤1	2	0	0	
		1~2				
		≥2				
	Farmhouse 2	New Zealand white rabbit	6	0	0	
	Age(years)*	≤1	6	0	0	
		1~2				
		≥2				
Zhoukou	Farm 3	Anyang grey rabbit	97	12	12	ST7 (12)
	Age (years)	≤1	93	12	13	ST7 (12)
		1~2	3	0	0	
		≥2	1	0	0	
		Chinese black rabbit	1	0	0	
	Age (years)	≤1	1	0	0	
		1~2				
		≥2				
	Farmhouse 3	New Zealand white rabbit	30	0	0	
	Age (years)*	≤1	21	0	0	
		1~2				
		≥2				
	Pet shop	Dutch-belted rabbit	1	0	0	
	Age (years)	≤1	1	0	0	
		1~2				
		≥2				
		Pygmy rabbit	3	0	0	
	Age (years)	≤1	3	0	0	
		1~2				
		≥2				
Total			231	21	9	ST1 (4), ST3 (5), ST7 (12)

[a]Some rabbit breeds cannot be counted. *Some age information cannot be counted.

and ST2 (Parkar et al. 2010). Children and monkeys in Nepal were infected with *Blastocystis* subtype ST2 (Yoshikawa et al. 2009). Australian pig farm workers and their pigs were infected with *Blastocystis* subtype ST5 (Wang et al. 2014). Nepalese breeders and their cattle and goats were infected with *Blastocystis* subtype ST6 (Lee et al. 2012a,b).

Fig. 1. Geographical distribution of infection with *Blastocystis* sp. in domestic rabbits in Henan, Central China. Infection rate expressed as prevalence.

These findings indicate the possibility of transmission of *Blastocystis* sp. between humans and animals.

China is the world's largest rabbit farmer in the world, and rabbit meat consumption is an important part of China's economy (Li et al. 2018). Infection with *Blastocystis* sp. can seriously threaten the health of rabbits and lead to economic losses to the rabbit industry (Li et al. 2020). Domestic rabbits are in close contact with humans, and they easily spread and infect humans through faecal-oral transmission after *Blastocystis* sp. infection. At present, research on the prevalence and subtype diversity of *Blastocystis* sp. in domestic rabbits is very limited. Only two subtypes, ST4 and ST14, have been identified in rabbits (Wang et al. 2018a, AbuOdeh et al. 2019, Li et al. 2020). Therefore, this study aims to determine the prevalence and subtype distributions of *Blastocystis* sp. in domestic rabbits of Henan Province in Central China, and to provide a basis for evaluating the zoonotic potential of *Blastocystis* sp. from rabbits in the future.

Table 2. Prevalence and subtypes of *Blastocystis* sp. in domestic rabbits in Henan Province, Central China.

Variable	Factors	No. tested	No. positive	Prevalence (%)[#]	95% CI	Subtype (n)
Breeds[a]	New Zealand white rabbit	82	7	9^{A}	2–15	ST1 (3), ST3 (4)
	Chinese black rabbit	5	2	40^{B}	-28–108	ST1 (1), ST3 (1)
	Anyang grey rabbit	97	12	12^{AB}	6–19	ST7 (12)
	Bluish blue rabbits	1	0	0		
	Harbin white Rabbit	2	0	0		
	Belgian hare	24	0	0		
	Dutch-belted rabbit	17	0	0		
	Pygmy rabbit	3	0	0$_{C}$		
Region	Shangqiu	16	8	50$_{A}$	23–78	ST1 (3), ST3 (5)
	Zhumadian	75	1	1$_{C}$	0–4	ST1 (1)
	Anyang	63	22	35$_{B}$	23–47	ST1 (22)
	Zhoukou	132	12	9	4–14	ST7 (12)
Age (years)[*]	≤1	195	30	15^{A}	10–21	ST1 (14), ST3 (4), ST7 (12)
	1~2	5	1	20^{AB}	0–76	ST1 (1)
	≥2	5	4	80^{B}	25–136	ST1 (4)
Total		286	43	15		ST1 (26), ST3 (5), ST7 (12)

[a]Some rabbit breeds cannot be counted; [#]Values with different superscript (A, B, C) in the same column are significantly different ($P < 0.05$); [*]Age of somerabbits was not available.

MATERIALS AND METHODS

Ethical statements

In the current study, all of the protocols obtained the review and approval of the Ethical Commission of the Xinxiang Medical University (No. XYLL-2019B007).

Sampling

From April to June 2020, 286 domestic rabbit faecal samples, including New Zealand white rabbit, Chinese black rabbit, Anyang grey rabbit, bluish blue rabbits, Harbin white rabbit, Belgian hare, Dutch-belted rabbit and Pygmy rabbit, were collected from three farms (n = 222), a pet shop (n = 4) and a pet trading market (n = 60) in four areas of Henan Province (Table 1). Among 286 domestic rabbits faecal samples, there were 81 samples with unknown age information, and 205 samples with age information were analysed (Table 2). Domestic rabbit faecal samples were collected with sterile gloves from cages. At the time of sampling, all rabbits examined appeared in good health and no diarrhea was observed. There were 16 samples from Shangqiu, 75 samples from Zhumadian, 63 samples from Anyang and 132 samples from Zhoukou. We collected approximately 1 g of faeces from each rabbit and put each faecal sample into a 15 ml sampling tube and labelled it, and record the breed and age of the domestic rabbits. The ages of the rabbits with collected stool samples ranged from two months to 24 months (Table 1). Faeces were stored at 4 °C and sent to the laboratory for testing within 24 hours.

DNA extraction and PCR detection

Genomic DNA was extracted from ~200 mg stool samples using a TIANamp Stool DNA Kit (TIANGEN, Beijing, China) in accordance with the manufacturer's instructions. Extracted DNA was stored at -80 °C until PCR analysis. All samples were screened for the presence of *Blastocystis* sp. by polymerase chain reaction (PCR) amplification of the barcode region (a fragment of ~600 bp) of the SSU rRNA gene using the primers BhRDr (5′-GAGCTTTTTAACTGCAACAACG-3′) and RD5 (5′-ATCTGGTTGATCCTGCCAGT-3′) (Scicluna et al. 2006). Each 25 µl PCR system contained 12.5 µl of 2xTaq Plus Master Mix (Vazyme, Nanjing, China), 8.5 µl of ddH_2O, 1 µl of each primer (10 µM), and 2 µl of genomic DNA. The amplification conditions were as follows: predenaturation at 95 °C for 5 min, denaturation

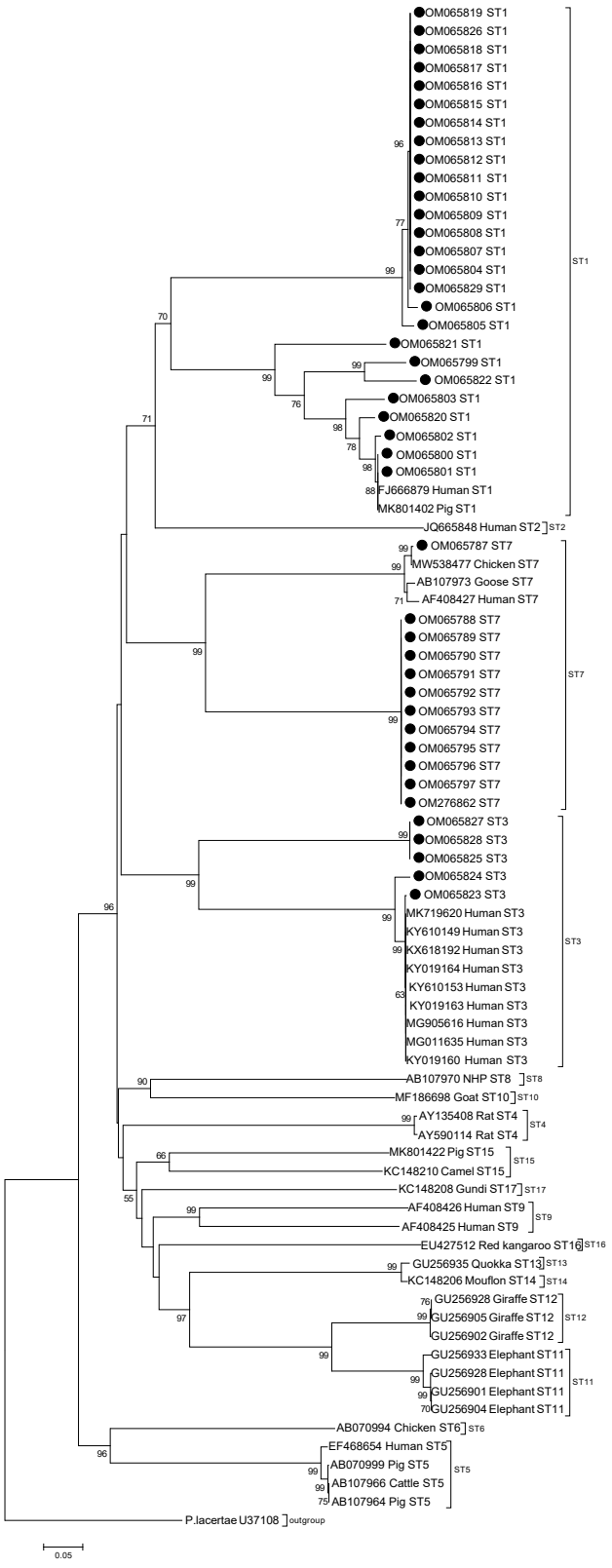

Fig. 2. Phylogenetic relationships among SSU rRNA gene sequences of *Blastocystis* sp. The phylogenetic tree shown was the constructed using the neighbor-joining method. Accession numbers for the sequences used are shown, followed by the common name of the host in English and the subtypes that the sequences belong to, only values above 50% are shown. The sequences obtained in this study are indicated by filled circles.

at 94 °C for 1 min, annealing at 59 °C for 1 min, and extension at 72 °C for 1 min; these conditions were repeated for 30 cycles, with a final extension at 72 °C for 7 min. All PCR products were visualised with 1% agarose gel electrophoresis with Gold View staining (Solarbio, Beijing, China). The positive PCR products were sent to Wuhan GeneCreate Biological Engineering Co., Ltd., Wuhan, China, for bidirectional sequencing.

Phylogenetic analysis

The subtypes of the Henan Province isolates were identified through BLAST search in GenBank by determining the exact match or closest similarity against known *Blastocystis* sp. subtypes. Sequence alignment using the ClustalW function of MEGA 7.0 software (http://www.megasoftware.net/) and by ocular inspection to remove gaps and ambiguous sequences. Then, the phylogenetic tree construction of positive SSU rRNA gene sequences of *Blastocystis* sp. was carried out using the neighbor-joining (NJ) method in Mega 7.0 (Saitou and Nei 1987). The Kimura 2-PA-Rameter model and bootstrap analysis (1000 replicates) were used to evaluate the reliability of the phylogenetic tree (Kumar et al. 2018). The nucleotide sequences obtained in this study have been deposited in GenBank under the accession numbers: OM065787–OM065797, OM065799–OM065829, OM276862.

Statistical analysis

All statistical analyses were carried out by SPSS 20.0 software (SPSS Inc., Chicago, USA). The infection rates of *Blastocystis* sp. in different areas, ages and breeds of domestic rabbits were tested by the chi-square test, and the differences in *Blastocystis* sp. infection rates among these different variables were analysed. $P < 0.05$ was considered statistically significant.

RESULTS

Infection rates of *Blastocystis* sp.

Out of the 286 faecal samples collected from eight different breeds of domestic rabbits, 15% (43/286) of the samples were positive for *Blastocystis* sp. The occurrence of *Blastocystis* sp. in New Zealand white rabbits, Chinese black rabbits and Anyang grey rabbits were 9% (7/82), 40% (2/5) and 12% (12/97), respectively. We did not detect *Blastocystis* sp. in the faecal samples of other breeds of rabbits. The infection rate of *Blastocystis* sp. in Chinese black rabbits was higher than that in New Zealand white rabbits ($P < 0.05$, Table 2). There was no significant difference in the infection rate of *Blastocystis* sp. between Anyang grey rabbits and New Zealand white rabbits or Chinese black rabbits ($P > 0.05$, Table 2).

The *Blastocystis* sp. infection rate was highest in rabbits aged ≥ 2 years (80%, 4/5), followed by rabbits aged 1–2 years (20%, 1/5), and the lowest infection rate occurred in rabbits aged ≤ 1 year (15%, 30/195) (Table 2). Rabbits aged ≥2 years had significantly higher *Blastocystis* sp. prevalence (80%, 4/5) compared with rabbits aged ≤1 year (15%, 30/195) ($P < 0.05$).

As shown in Fig. 1 and Table 2, the prevalence of *Blastocystis* sp. in different investigated areas ranged from 1% to 50%. The highest prevalence of *Blastocystis* sp. was in Shangqiu (50%, 8/16), followed by Anyang (35%, 22/63)

Table 3. Prevalence and subtypes of *Blastocystis* sp. found in rabbits in different countrie.

Country	Host	No. tested	No. positive	Prevalence (%)	Subtype (n)	Reference
United Arab Emirates	Rabbit	3	1	33	ST14 (1)	AbuOdeh et al. 2019
China	New Zealand white rabbit	215	7	3	ST4 (7)	Wang et al. 2018a
China	New Zealand rabbit, Long-haired rabbit, Tolai hare	616	6	1	ST4 (6)	Li et al. 2020
China	New Zealand white rabbit, Chinese black rabbit, Anyang grey rabbit, Bluish blue rabbits, Harbin White Rabbit, Belgian hare, Dutch-belted rabbit, Pygmy Rabbit	286	43	15	ST1 (26), ST3 (5), ST7 (12)	This study

and Zhoukou (9%, 12/132), and the lowest in Zhumadian (1%, 1/75). Faecal samples of domestic rabbits collected from Zhoukou, Anyang and Shangqiu were likely to be positive for *Blastocystis* sp. than those collected from Zhumadian ($P < 0.05$).

Identification of *Blastocystis* subtypes

The sequencing analysis of *Blastocystis* sp. of domestic rabbits from Henan Province in Central China based on SSU rRNA gene loci revealed that 26 sequences were located on the same clade as *Blastocystis* ST1, five sequences were located on the same clade as *Blastocystis* ST3 and 12 sequences were located on the same clade as *Blastocystis* ST7 in the GenBank database (Fig. 2). Three genotypes, ST1 (26/43, 60%), ST3 (5/43, 12%) and ST7 (12/43, 28%), were detected in this study, all of which were zoonotic genotypes. Among them, ST1 was the dominant subtype of *Blastocystis* sp. (Table 2).

Distribution of *Blastocystis* subtypes

As shown in Table 2, ST1 (n = 22) was detected in domestic rabbit fecal samples from Anyang, while ST1 (n = 3) and ST3 (n = 5) were detected in Shangqiu, ST7 (n = 12) was detected in Zhoukou, and ST1 (n = 1) was detected in Zhumadian. ST1 and ST3 were found in New Zealand white rabbits and Chinese black rabbits, showing a relatively wide distribution. The zoonotic subtype of Anyang grey rabbit was ST7. Based on the analysis of subtypes of *Blastocystis* sp. in domestic rabbits of different ages, it was found that three zoonotic subtypes ST1, ST3 and ST7 were found in rabbits aged ≤ 1 year. The zoonotic subtypes of domestic rabbits infected with aged 1–2 years and ≥ 2 years were all ST1.

DISCUSSION

At present, there are few studies on the infection and subtypes of *Blastocystis* sp. in rabbits (Table 3). Rabbits infected with *Blastocystis* ST4 were reported in Heilongjiang, Liaoning, Jilin Provinces (Wang et al. 2018a) and Shandong Province (Li et al. 2020) of China, and ST14 has been reported in the United Arab Emirates (AbuOdeh et al. 2019). Further understanding of the infection and subtypes of *Blastocystis* sp. in rabbits in other areas of China will provide data support for future prevention strategies and evaluation of zoonotic potential of *Blastocystis* sp. in rabbits.

In the present study, the prevalence of *Blastocystis* sp. in domestic rabbits in different areas of Henan Province in Central China was reported for the first time. The infection rate of *Blastocystis* sp. in rabbits in Heilongjiang, Liaoning and Jilin Provinces, China (3%, 7/215) (Wang et al. 2018a) and in Shandong Province, China (1%, 6/616) (Li et al. 2020) are lower than that in this study. However, the infection rate of *Blastocystis* sp. in domestic rabbits in the present study was lower than that in United Arab Emirates rabbits (one of three rabbits infected) (AbuOdeh et al. 2019) (Table 3). These differences may be due to different ecological conditions, climate, survey period, sample size and rabbit species.

The infection rate of *Blastocystis* sp. was the highest (40%, 2/5) in Chinese black rabbits, followed by 12% (12/97) of Anyang grey rabbits and 9% (7/82) of New Zealand white rabbits. Prevalence rate of *Blastocystis* sp. in Chinese black rabbits and Anyang grey rabbits was higher than that in New Zealand white rabbits, which may be due to the fact that New Zealand white rabbits were mostly raised separately in farmhouses and pet markets, while Chinese black rabbits and Anyang grey rabbits are reared centrally in farms. Compared with New Zealand white rabbits, the feeding environment of Chinese black rabbits and Anyang grey rabbits is relatively unclean and unhygienic, and they have more opportunities to contact the source of *Blastocystis* sp. infection.

The highest infection rate of *Blastocystis* sp. was found in rabbits aged ≥ 2 years, followed by rabbits aged 1–2 year, and rabbits aged ≤ 1 year. This may be related to the increase of age, because rabbits are more likely to be exposed to *Blastocystis* sp. contamination and more likely to be infected with *Blastocystis* sp. (Calvete et al. 2018).

According to the available data, only two subtypes of *Blastocystis* sp. were identified in rabbits, ST4 and ST14 (Wang et al. 2018a, AbuOdeh et al. 2019, Li et al. 2020), among which ST4 was potentially zoonotic. In this study, three subtypes of *Blastocystis* sp. (ST1, ST3 and ST7) were identified, all of which were potentially zoonotic. These results suggest that rabbits may be the potential source of human infection with *Blastocystis* sp., and prevention and control measures should be taken. So far, five subtypes (ST1, ST3, ST4, ST7 and ST14) of *Blastocystis* sp. have been identified in rabbits.

To the best of our knowledge, this is the first time that ST1, ST3 and ST7 have been reported in rabbits. However, due to the limited data, it is not possible to determine the dominant or specific subtypes of *Blastocystis* sp. in rabbits. Existing studies have shown that ST1 and ST3 were the main subtypes of *Blastocystis* sp. in humans around the world, including China (Jantermtor et al. 2013, Coskun et al. 2016, Khademvatan et al. 2017, Zhang et al. 2017, Melo et al. 2019, Kim et al. 2020). A recent study has shown that the main subtypes of *Blastocystis* sp. infection in hospital patients in central China are also ST1, ST3 and ST7 (Li et

al. 2021). Therefore, attention should be paid to the transmission of *Blastocystis* sp. between humans and rabbits in central China. In addition, *Blastocystis* subtypes ST1, ST3 and ST7 have also been found in Chinese goats, sheep, domestic dogs, Arctic foxes and crab-eating monkeys (Zanzani et al. 2016, Song et al. 2017a,b, Wang et al. 2018b), in goats from Malaysia (Tan et al. 2013) and in cattle from Lebanon (Greige et al. 2019). *Blastocystis* subtypes ST1, ST3 and ST7 have been found in both human and animal hosts, indicating that these subtypes have the potential to spread zoonosis.

In conclusion, this study first reports the prevalence of *Blastocystis* sp. in domestic rabbits in different areas of Henan Province, Central China, and the discovery of ST1, ST3 and ST7 of *Blastocystis* sp. in domestic rabbits. The discovery of zoonotic subtypes ST1, ST3 and ST7 in domestic rabbits suggests that domestic rabbits can potentially transmit *Blastocystis* sp. to humans and other animals in this area. Therefore, it is necessary to carry out corresponding research on the infection and subtype distribution of *Blastocystis* sp. in domestic rabbits and their breeders in other provinces of China, which will help to clarify the transmission mechanism and provide a basis for the formulation of effective strategies to prevent the occurrence of human *Blastocystis* sp. infections.

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