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Predominance of the *Blastocystis* subtype ST5 among free-living sympatric rodents within pig farms in China suggests a novel transmission route from farms

Fa Shan [a], Fang Wang [b,c,d], Shengke Chang [b,c,d], Nanhao Wang [b,c,d], Yufeng Liu [b,c,d], Xi Chen [a], Guanghui Zhao [a,*], Longxian Zhang [b,c,d,*]

[a] College of Veterinary Medicine, Northwest A&F University, Yangling 712100, China
[b] College of Veterinary Medicine, Henan Agricultural University, Zhengzhou 450046, China
[c] Key Laboratory of Quality and Safety Control of Poultry Products (Zhengzhou), Ministry of Agriculture and Rural Affairs, Zhengzhou 450046, China
[d] International Joint Research Laboratory for Zoonotic Diseases of Henan, Zhengzhou 450046, China

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ABSTRACT

Blastocystis is a parasitic protist that can infect humans and various domestic and wild animals. However, there is limited research on the prevalence of this parasite among rodents, particularly those living in pig farm settings. Therefore, to investigate the occurrence, molecular characterization, and zoonotic potential of *Blastocystis* among rodents within pig farm environments, we conducted an investigation of 227 rodents and shrews from 34 pig farms located in Henan, Shaanxi, and Shanxi provinces of China using nested PCR of the SSU rRNA gene of *Blastocystis*. The potential transmission and public health implications were also assessed from a One Health perspective. *Blastocystis* was detected in 86 (37.9%) fecal samples. The highest infection rate was observed among *Ruttus norvegicus* (73.7%, 42/58), followed by *Ruttus tanezumi* (30.1%, 41/136), and *Mus musculus* (12.0%, 3/25). However, it was not detected among individuals with *Apodemus agrarius* ($n = 1$) and *Crocidura shantungensis* ($n = 7$). Five known zoonotic *Blastocystis* subtypes (ST1–ST5) were identified, with ST4 (51.2%, 44/86) and ST5 (40.7%, 35/86) being the predominant ones, followed by ST1 (3.5%, 3/86), ST3 (3.5%, 3/86), and ST2 (1.2%, 1/86). ST4 was prevalent among *R. norvegicus* (83.3%, 35/42), while ST5 dominated *R. tanezumi* (70.7%, 29/41). Furthermore, ST5 exhibited the widest distribution at pig farm level, accounting for 65.0% (13/20) of *Blastocystis*-positive pig farms. This investigation presents the first documented *Blastocystis* infection in *R. tanezumi* and *M. musculus*, highlighting the predominant presence of the zoonotic ST5 subtype in rodents for the first time. The results demonstrate that sympatric rodents can serve as natural reservoirs for *Blastocystis* and play a role in its transmission. These findings provide information on the dynamics of rodent transmission and emphasize the potential public health threat posed by zoonotic *Blastocystis* subtypes spillover from pig farms.

1. Introduction

Blastocystis, a commonly found gastrointestinal parasite belonging to the Stramenopile group of Heterokonts, is characterized as an anaerobic protist [1–3]. Transmission of this protist is thought to occur mainly through the fecal–oral route, either via direct contact with infected hosts or by ingesting contaminated food or water [4–6]. Numerous subclinical and asymptomatic cases of *Blastocystis* infection in humans and animals led to inquiries about its pathogenicity [1,7]. However, this parasite has been associated with gastrointestinal dysfunction, including symptoms such as diarrhea, nausea, and abdominal pain [7–9]. Crucially, *Blastocystis* colonization or infection, which affects an estimated one billion individuals and has been extensively documented among HIV/AIDS patients [10,11], highlighting its importance as a zoonotic pathogen that demands attention.

The occurrence of *Blastocystis* among different pets, livestock, and wildlife shows that these reservoir species have significant opportunities in transmitting the parasite to humans [2,9,12,13]. Epidemiological investigations using PCR-based analysis of the small subunit ribosomal RNA (SSU rRNA) gene have revealed substantial genetic variation

* Corresponding author at: College of Veterinary Medicine, Henan Agricultural University, No. 15 Longzihu University Area, Zhengdong New District, Zhengzhou 450046, China.
E-mail address: zhanglx8999@henau.edu.cn (L. Zhang).

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within *Blastocystis* [14]. The current classification of *Blastocystis* comprises 32 subtypes (STs), specifically ST1–ST32 [5,15,16]. Nonetheless, only 28 subtypes (ST1–ST17, ST21, and ST23–ST32) meet the existing criteria for individual subtype identification and are considered valid [5,14–16]. In humans, a total of 16 *Blastocystis* subtypes (ST1–ST10, ST12, ST13 ST14, ST16, ST23, and ST24) have been identified worldwide [9,12,17–19], with the majority of cases (over 95%) caused by ST1–ST4 [18]. However, a meta-analysis conducted in China revealed that Chinese human samples exhibited eight subtypes (ST1–ST7, and ST12), with ST1–ST3 being the most prevalent. Among patients with diarrhea, ST1 was the predominant subtype, while ST3 was most common in asymptomatic infections [55]. *Blastocystis* infection in domesticated pigs has been documented globally, with an overall incidence rate of 52.4%. The infection involves eight zoonotic subtypes (ST1–ST7, ST10) and one subtype specifically adapted to animals (ST15), where ST5 is the prevailing subtype [25]. Studies conducted on pigs in certain regions of China have indicated the presence of ST1, ST3, ST5, ST10, and mixed infections, with ST5 being the dominant subtype [25,62,63]. The presence of identical *Blastocystis* subtypes in both humans and animals, coupled with the similarity or exact match in their nucleic acid sequences, suggests a potential for zoonotic transmission [5,9,12,13,17–21].

Rodents and shrews exhibit high adaptability and a wide distribution, because of which they can thrive in diverse environments [22,23]. These wild small mammals are frequently found near humans and domestic pigs in pig farm settings, which increases the risk of humans and domestic pigs contracting infections from wildlife-derived zoonotic pathogens, and vice versa [22,24]. However, there are no available data regarding *Blastocystis* infection and subtype distribution in rodent species within pig farm settings. This lack of knowledge hinders our understanding of how these wild small mammals transmit *Blastocystis* to humans and pigs within pig farms. Consequently, the present study aims to investigate and confirm the prevalence and distribution of the *Blastocystis* infection among free-living sympatric rodents and shrews in Chinese pig farms, while also discussing potential transmission and public health implications.

2. Materials and methods

2.1. Sampling

Between March 2021 and June 2023, a total of 227 wild small mammals, including 136 Asian house rats (*Rattus tanezumi*), 58 brown rats (*Rattus norveqicus*), 25 house mouse (*Mus musculus*), one striped field mouse (*Apodemus agrarius*), and seven Asian lesser white-toothed shrews (*Crocidura shantungensis*), were trapped from 34 pig farms located in Henan (Anyang, Jiaozuo, Kaifeng, Luohe, Luoyang, Nanyang, Pingdingshan, Puyang, Shangqiu, Xuchang, Xinxiang, Xinyang, Zhoukou, Zhumadian, and Zhengzhou), Shaanxi (Baoji and Weinan), and Shanxi (Taiyuan) provinces in China (Fig. 1 and Table S1). All of the pig

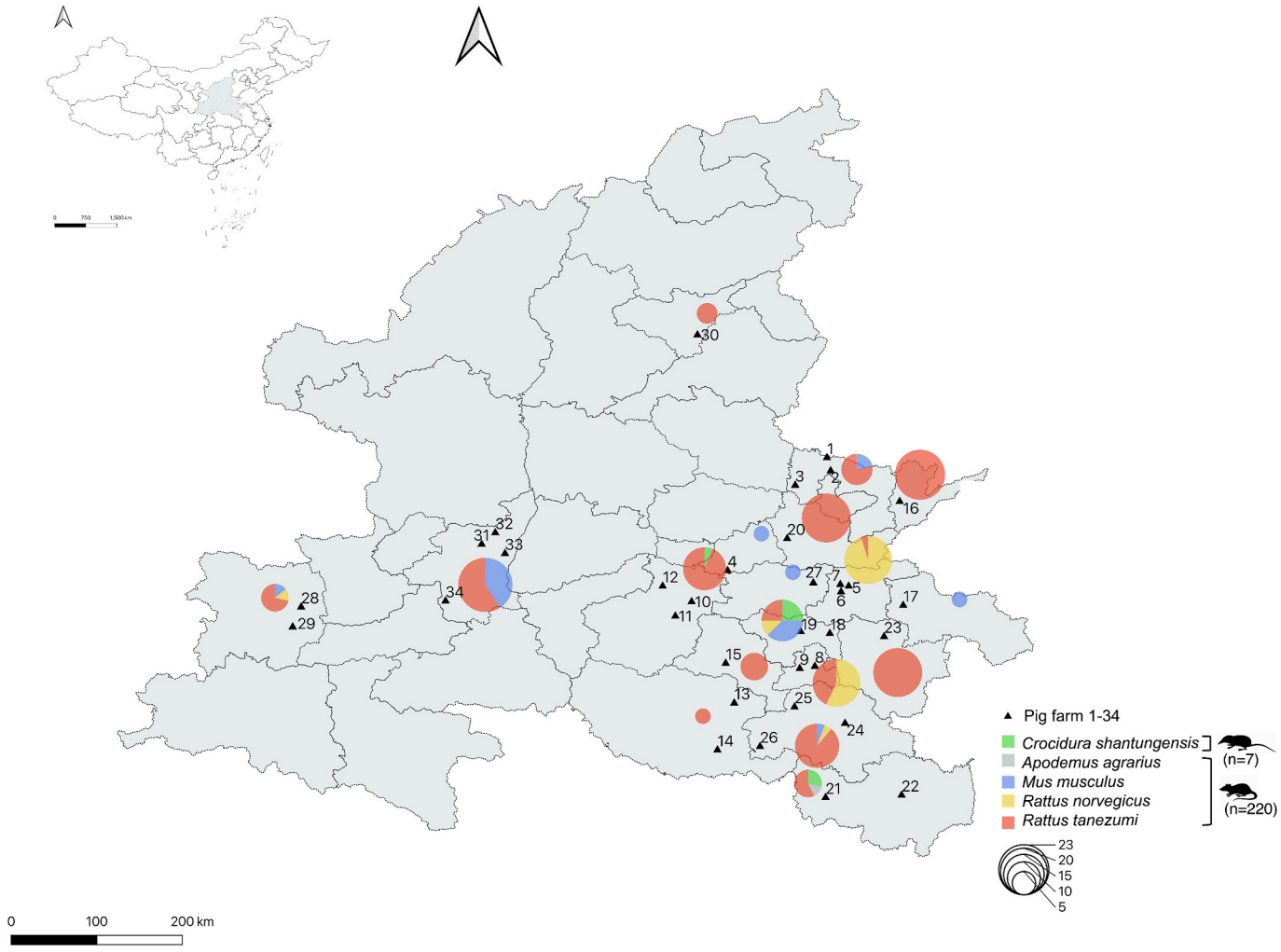

Fig. 1. Geographical distribution of sampling sites and number of animal samples collected in the present study. Each black triangle represents a sampling pig farm. The numbers of the 227 samples belonging to the five identified species are indicated by a pie chart for each city.

farms included in this study were large-scale breeding operations, and the captured wild small mammals ranged from 1 to 23 per farm (Table S1). These wild small mammals were humanely euthanized by CO_2 inhalation and then individually placed in bags marked with essential information, such as the sampling date, sampling site, farm size, farm type, and duration of pig rearing. Afterward, they were transported to the laboratory in 48 h using containers with ice packs for necropsy within a biosafety cabinet. The sex and body mass of each animal were documented before dissection. Fecal and liver samples were collected and preserved at −80 °C for further molecular analysis.

2.2. Genomic DNA extraction

Genomic DNA was extracted from the fecal sample and liver tissue of each wild small mammal using the QIAamp PowerFecal Pro DNA Kit (Qiagen, Hilden, Germany) and the TIANamp Genomic DNA Kit (Tiangen, Beijing, China), respectively. The manufacturer's instructions were followed for both extraction methods. The concentration and quality of the extracted DNA were measured using the NanoDrop One Spectrophotometer. The successfully extracted samples were labeled and stored at −20 °C for future molecular analysis.

2.3. PCR amplification

The identification of wild small mammal species was achieved by employing PCR-based amplification of the cytochrome *b* (cytb) gene. The PCR conditions and primer design complied with the protocol established by Nicolas, leading to a PCR product size of 1100–1200 bp [27,28].

The presence of *Blastocystis* in fecal genomic DNA was detected using nested PCR amplification targeting a 479-bp fragment of the SSU rRNA gene. The PCR conditions and the external/internal primers were as previously documented [19,29]. Each PCR assay had positive (ST6 from humans) and negative (ultrapure H2O) controls. The amplicons obtained from the secondary PCR were stained with SYBR Green reagent (Tiandz, Inc., Beijing, China) and analyzed using 1.0% agarose gel electrophoresis to confirm the presence of the target DNA fragment.

2.4. Sequencing and sequence analysis

Following the guidelines provided by the Cycle Sequencing Kit (BigDye Terminator v3.1), the ABI 3730XL DNA Analyzer (Applied Biosystems, Thermo Fisher Scientific, Foster City, CA USA) was used to conduct sequencing in both directions for the positive amplicons obtained from each sample. To ensure the accuracy of the identified nucleotides, the raw nucleotide sequences were corrected and assembled using DNAStar 7.1 (https://www.dnastar.com/). The assembled clean sequences were compared with GenBank sequences using BLAST analysis (http://www.ncbi. nlm.nih.gov/BLAST/) and the *Blastocystis* definitions database (https://pubmlst.org/bigsdb?db=pubmlst_blastocystis_seqdef). Subsequently, the obtained sequences were aligned with the reference sequences obtained from GenBank. Finally, a phylogenetic evolutionary tree was built using the neighbor-joining (NJ) method and the Kimura 2-parameter model with 1000 bootstrap replicates in MEGA X software (http://www.megasoftware.net/).

2.5. Statistical analysis

Statistical analysis was performed using IBM SPSS 26.0 for Mac (IBM Corp., Armonk, NY, USA). The level of the correlation between *Blastocystis* infection and the variables was evaluated by computing the odds ratio (*OR*) and 95% confidence interval (*CI*) through either the chi-squared test or Fisher's exact test. The risk factors associated with *Blastocystis* infections were evaluated using univariate and multivariate logistic regression analyses. Statistical significance was determined at a *P*-value of <0.05.

3. Results

3.1. Prevalence of Blastocystis

The prevalence of *Blastocystis* was 37.9% (86/227) based on nested PCR amplification of the SSU rRNA gene. The highest prevalence was observed in *R. norvegicus* (72.4%, 42/58), followed by *R. tanezumi* (30.1%, 41/136), and *M. musculus* (12.0%, 3/25; Table 1), with a statistically significant difference between them ($\chi2 = 37.934$, $P < 0.001$; Table 2). However, the individuals of *A. agrarius* and *C. shantungensis* were all negative for *Blastocystis*. When considering the regional distribution, there was a statistically significant difference ($\chi2 = 8.914$, $P = 0.008$) in *Blastocystis* infection between the three provinces included in this study, with a prevalence of 75.0%, (3/4), 40.7% (11/189) and 17.8% (6/34) for the provinces of Shanxi, Henan, and Shaanxi, respectively (Table 2). To ensure statistical accuracy, the data from Shanxi province were excluded from the analysis due to its small sample size. A re-analysis of the infection rates between Henan and Shaanxi provinces was then perfomed, and the results confirmed that there was also a statistically significant difference in prevalence between the two provinces ($\chi2 = 6.577$, $P = 0.01$). In addition, *Blastocystis* was found in 20 of the 34 pig farms surveyed, resulting in a farm-level positivity rate of 58.8% (95% *CI*: 51.68–60.18) (Table S1).

3.2. Risk factors for Blastocystis infection

Univariate analysis demonstrated a connection between *Blastocystis* infection and region, host species, and duration of pig rearing (Table 2). However, factors such as host age, host sex, season, farm type, and farm size did not influence *Blastocystis* infection. Further analysis using the variables included in the multivariate model (region, host species, duration of pig rearing) identified two factors significantly associated with *Blastocystis* infection: host species and duration of pig rearing (Table 2).

3.3. Distribution of Blastocystis subtypes

Five known zoonotic subtypes (ST1–ST5) were identified among the 86 *Blastocystis* isolates in this study (Table 1). ST4 and ST5 were the predominant subtypes, accounting for 51.2% (44/86) and 40.7% (35/86), respectively. The other three subtypes occurred only occasionally: ST1 (3.5%, 3/86), ST2 (1.2%, 1/86), and ST3 (3.5%, 3/86; Table 1). Among the three *Blastocystis*-positive rodent species, four subtypes (ST1, ST2, ST4, and ST5) were found in 42 *R. norvegicus* samples, with ST4 ($n = 35$) being the most prevalent; four subtypes (ST1, ST3, ST4, and ST5) were found in 40 *R. tanezumi* samples, with ST5 ($n = 29$) being the dominant subtype; and three subtypes (ST1, ST4, and ST5) with even

Table 1
Occurrence and subtype distribution of *Blastocystis* in rodents and shrews from pig farms in China.

Host Species	No. Tested (%)	No. Positive (%)	Subtypes (No.)
Asian house rat (*Rattus tanezumi*)	136 (59.9%)	41 (30.1%)	ST1(2), ST3(2), ST4(8), ST5(29)
Brown rat (*Rattus norvegicus*)	58 (25.6%)	42 (72.4%)	ST1(1), ST2(1), ST4(35), ST5(5)
House mouse (*Mus musculus*)	25 (11.0%)	3 (12.0%)	ST3(1), ST4(1), ST5(1)
Striped field mouse (*Apodemus agrarius*)	1 (0.4%)	0 (0%)	\
Asian lesser white-toothed shrew (*Crocidura shantungensis*)	7 (3.1%)	0 (0%)	\
Total	227	86 (37.9%)	ST1(3), ST2(1), ST3(3), ST4(44), ST5(35)

Table 2
Risk factors for *Blastocystis* infection in rodents and shrews within pig farms.

Variable		No. tested	No. positive	positive rate % (95% *CI*)	Univariate analysis		Multivariate analysis	
					P-value	*OR* (95% *CI*)	*P*-value	*OR* (95% *CI*)
Host Species	*Rattus tanezumi*	136	41	30.1 (22.3–38.0)	< 0.001	3.17 (0.90–11.17)	< 0.001	3.08 (0.85–11.17)
	Rattus norvegicus	58	42	72.4 (60.6–84.3)		19.25 (5.06–73.27)		17.15 (4.25–69.17)
	Mus musculus	25	3	12.0 (−1.7–25.2)		1		1
	Others	8	0	0 (−)		–		–
Host Age	Juvenile	79	28	35.4 (24.7–46.2)	0.579	1		
	Adult	148	58	39.2 (31.2–47.1)		1.17 (0.67–2.07)		
Host Sex	Female	110	46	41.8 (32.5–51.2)	0.236	1.38 (0.81–2.37)		
	Male	117	40	34.2 (25.5–42.9)		1		
Region	Henan	189	77	40.7 (33.7–47.8)		3.21 (1.27–8.12)		
	Shaanxi	34	6	17.6 (4.1–31.1)	0.008	1		
	Shanxi	4	3	75.0 (−4.6–154.6)		14 (1.23–158.84)		
Season	Spring	135	51	37.8 (29.5–46.1)		2.13 (0.43–10.63)		
	Summer	54	23	42.6 (29–56.2)	0.646	2.6 (0.49–13.68)		
	Autumn	9	2	22.2 (−11.7–56.1)		1		
	Winter	29	10	34.5 (16.1–52.9)		1.84 (0.32–10.58)		
Farm type	Breeding farm	166	61	36.7 (29.3–44.2)	0.560	1		
	Fattening farm	61	25	41.0 (28.3–53.7)		1.2 (0.66–2.18)		
Farm size	> 10,000 pigs	99	45	45.5 (35.5–55.4)		1.81 (0.64–5.13)		
	2000–10,000 pigs	109	35	32.1 (23.2–41)	0.118	1.03 (0.36–2.92)		
	< 2000 pigs	19	6	31.6 (8.6–54.6)		1		
Duration of pig rearing	> 5 years	78	31	39.7 (28.6–50.8)		5.15 (1.83–14.49)		4.61 (1.54–13.75)
	3–5 years	105	50	47.6 (37.9–57.3)	< 0.001	7.09 (2.59–19.41)	0.023	3.67 (1.25–10.72)
	< 3 years	44	5	11.4 (1.6–21.1)		1		1

The "–" symbol indicates the data were not be calculated;
OR, Odd ratio; *CI,* Confidence interval.

distribution were found in three *M. musculus* samples (Fig. 2A). Although ST4 was the most abundant subtype, ST5 exhibited the widest distribution, accounting for 65.0% (13/20) of *Blastocystis*-positive pig farms (Fig. 2B). Mixed subtype infections were not observed in this study.

3.4. Phylogenetic and sequence analysis of Blastocystis subtypes

For sequence analysis, 86 *Blastocystis* isolates were sequenced, which yielded 15 representative sequences ranging from 468 to 484 bp. All the sequences obtained in this study exhibited a significant similarity to *Blastocystis* sequences previously registered on GenBank (Table 3). Among the isolates, three ST1 isolates produced three variations, of which ST1a was detected in *R. norvegicus*, while ST1b and ST1c were detected in *R. tanezumi*. Additionally, one ST2 isolate from a *R. norvegicus* sample produced the ST2a sequence, which had 100% homology with a human (*Homo sapiens*) sample (KU147354) from Mexico. Moreover, two variations were identified among three ST3 isolates, with ST3a found in two *R. tanezumi* samples and ST3b in one *M. musculus* sample. Interestingly, only one variation was identified among 44 ST4 isolates, of which 35 isolates were from *R. norvegicus*, eight were from *R. tanezumi*, and one from *M. musculus*.

In the case of ST5, 35 isolates produced eight variants (ST5a to ST5h). ST5a (n = 15) was detected in 14 *R. tanezumi* and one *R. norvegicus* with 100% homology to a pig sample (KF410601) from the USA. ST5b (n = 1) was found in a *R. tanezumi* with 100% homology to a pig sample (KF410606) from the USA. ST5c (n = 11) was detected in nine *R. tanezumi* and two *R. norvegicus* with 100% homology to a human sample (OP725977) from the USA. ST5d (n = 1) was detected in a *R. norvegicus* with 100% homology to a pig sample (MK801407) from

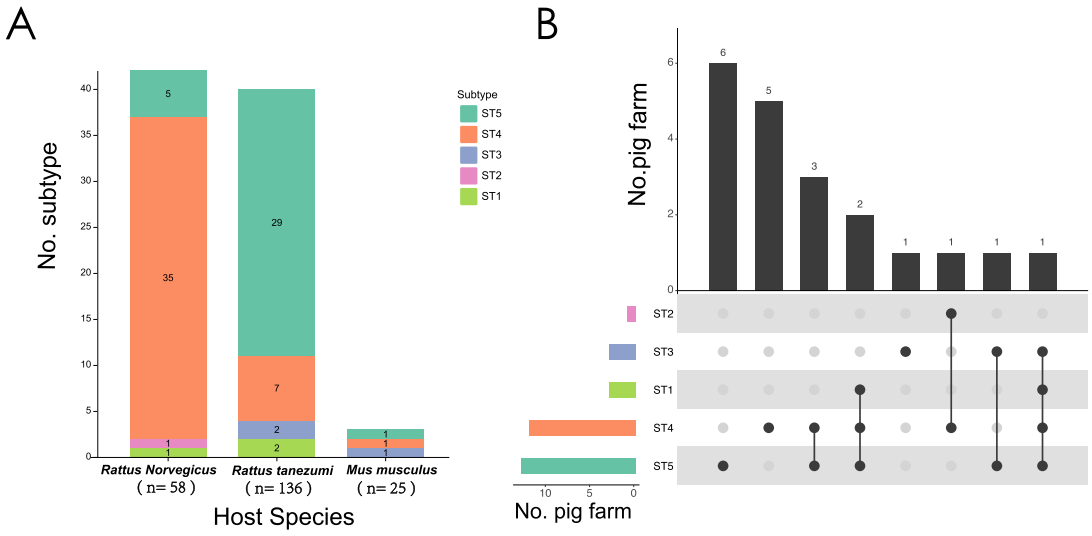

Fig. 2. Distribution and frequency of *Blastocystis* subtypes in the present study. **(A)** Distribution of *Blastocystis* subtypes among host species. **(B)** Frequency of *Blastocystis* subtypes among pig farms.

Table 3
Sequences/alleles of *Blastocystis* identified among rodents within pig farms.

Host species	Subtype/alleles (No.)	References (Homology %)	Nucleotide substitution positions	GenBank ID
Rattus tanezumi	ST1a/ allele 88 (1)	KU147329 (100%)		OR754903
	ST1b/ allele 4 (1)	OP725948 (100%)		OR754904
	ST3a/ allele 34 (2)	KU147331 (100%)		OR754905
	ST4a/ allele 92 (8)	OQ727432 (100%)		OR754906
	ST5a/ allele 119 (14)	KF410601 (100%)		OR754907
	ST5b/ allele 119 (1)	KF410606 (100%)		OR754908
	ST5c/ allele 119 (9)	OP725977 (100%)		OR754909
	ST5e/ allele 115 (3)	KF410604 (100%)		OR754910
	ST5g/ allele 115 (1)	MK375236 (100%)		OR754911
	ST5h/ allele 119 (1)	OP725974 (100%)		OR754912
	ST1c/ allele 2 (1)	KU147350 (99.8%)	269 (T to C)	OR754913
	ST2a/ allele 13 (1)	KU147354 (100%)		OR754914
	ST4a/ allele 92 (35)	OQ727432 (100%)		OR754915
Rattus norvegicus	ST5a/ allele 119 (1)	KF410601 (100%)		OR754916
	ST5c/ allele 119 (2)	OP725977 (100%)		OR754917
	ST5d/ allele 119 (1)	MK801407 (100%)		OR754918
	ST5e/ allele 115 (1)	KF410604 (100%)		OR754919
	ST3b/ allele 36 (1)	KU147393 (100%)		OR754920
Mus musculus	ST4a/ allele 92 (1)	OQ727432 (100%)		OR754921
	ST5f/ allele 115 (1)	ON394481 (99.8%)	135 (G to T)	OR754922

Germany. ST5e (n = 4) was detected in three *R. tanezumi* and one *R. norvegicus* with 100% homology to a pig sample (KF410604) from the USA. ST5f (n = 1) was detected in a *M. musculus* and showed 99.8% homology to a pig sample (KF410604) from the USA, with a nucleotide substitution at position 135 (G to T). ST5g (n = 1) was detected in a *R. tanezumi* with 100% homology to a pig sample (MK375236) from China. ST5h (n = 1) was detected in a *R. tanezumi* with 100% homology to a human sample (OP725974) from Colombia (Table 3).

The *Blastocystis* definition database was used to assign names to alleles, leading to the identification of three distinct alleles (allele 2, allele 4, and allele 88) within ST1, two distinct alleles (allele 34 and allele 36) within ST3, and two distinct alleles (allele 115 and allele 119) within ST5. ST2 displayed the presence of allele 13, while ST4 exhibited the presence of allele 92 (Table 3). Among the examined wild small mammals, allele 92 (51.2%, 44/86) and allele 119 (33.8%, 29/86) were the most prevalent *Blastocystis* alleles (Table 3).

Phylogenetic analysis clearly showed that the sequences of the five subtypes clustered with their respective reference subtype sequences. Fifteen representative sequences from this study and 49 reference sequences from the GenBank database were used for this analysis, with one outgroup included (Fig. 3).

4. Discussion

Blastocystis is a globally distributed zoonotic protist [2,10]. Although previous reports have concentrated mainly on *Blastocystis* infection in livestock and humans [12,18], limited information is available on its presence in rodents, particularly in farm settings. This study aims to fill this gap by presenting the occurrence of *Blastocystis* infection among free-living sympatric rodents and shrews within pig farms in China. At 37.9% (86/227), the overall infection rate in our study was higher than that in farmed rodents (10.2%) [19,26,30], free-living in community rodents (12.6%) [31,32], lab rodents (8.2%) [33], pet rodents (8.1%) [34–36], wild rodents (27.0%) [37–45], and zoo rodents (20.0%) [39,46–48], but lower than that of free-living sewer rodents (77.0%) [49] (Table S2). Variations in prevalence among these studies can be attributed to factors such as sample size, environmental sanitation, and susceptibility of rodent species to *Blastocystis* [12,14,18,25]. More research is needed to determine the factors that contribute to this variability. Furthermore, a significant prevalence (58.8%, 20/34) of *Blastocystis* infection was observed in the pig farms in the study areas. Given the high prevalence of *Blastocystis* as a parasite in domestic pigs, it is crucial to determine the potential transmission between pigs and these wild small mammals.

Approximately 22 rodent species have been surveyed for *Blastocystis* infection [12,25]. Our study identified positive results for *Blastocystis* infection in three common rodent species: *R. norvegicus*, *R. tanezumi*, and *M. musculus*. Among them, *R. norvegicus*, the largest commensal rodent species in the world, exhibited the highest prevalence of *Blastocystis* in our investigation, with a rate of 73.7% (42/58). This prevalence was higher than what was observed in brown rats in the community in Iran (15.8%) [31], in the wild in Japan (25.0%) [41], and in Malaysia (16.2%) [42], but similar to that found in sewer systems in Spain (77.0%) [49] (Table S2). These variations in prevalence could be due to differences in habitat sanitation conditions. Although *R. tanezumi* and *M. musculus* are also common rodent species worldwide [22], there have been no previous reports of *Blastocystis* infections in these two species. Hence, our study is the first to report the occurrence of *Blastocystis* infection in *R. tanezumi* and *M. musculus*.

In contrast, *Blastocystis* infection was absent in individuals of *A. agrarius* and *C. shantungensis*, which were only sporadically sampled in our study. Furthermore, no reports of *Blastocystis* infections in *A. agrarius* and *C. shantungensis* have been documented worldwide. The only test conducted for *Blastocystis* in shrews was in the house shrew (*Suncus murinus*) in Malaysia [42], which yielded a negative result. The differences observed in the occurrence of *Blastocystis* among the studied host species could be attributed to the restricted quantity of collected specimens and the susceptibility of the host species to *Blastocystis*. To better understand this phenomenon, further research is needed to expand the survey region and increase the sample size, which will help determine the susceptibility of species with small sample sizes to *Blastocystis*.

Geographical and environmental factors have been identified as potential determinants of the occurrence of *Blastocystis* prevalence in both animals and humans [4,50]. Our analysis of risk factors for *Blastocystis* infection showed that, apart from host species, the duration of pig rearing significantly affected the likelihood of acquiring *Blastocystis* infection among sympatric rodents living without *Blastocystis* within pig farms. Specifically, rodents in groups where pigs had been raised for >3 years were more susceptible to *Blastocystis* than those in groups where pigs had been raised for <3 years. Our evaluation of the correlation between duration of pig rearing and infection rates showed a positive correlation, with an *OR* of 3.67 (95% *CI*: 1.25–10.72) for the 3–5-year group and an *OR* of 4.61 (95% *CI*: 1.54–13.75) for the >5-year group, both compared to the <3-year group (Table 2). This association may be related to the environment, as these co-habiting rodents are chronically exposed to contaminated fecal matter in pig farms, increasing the likelihood of infection with *Blastocystis* [38]. This finding further

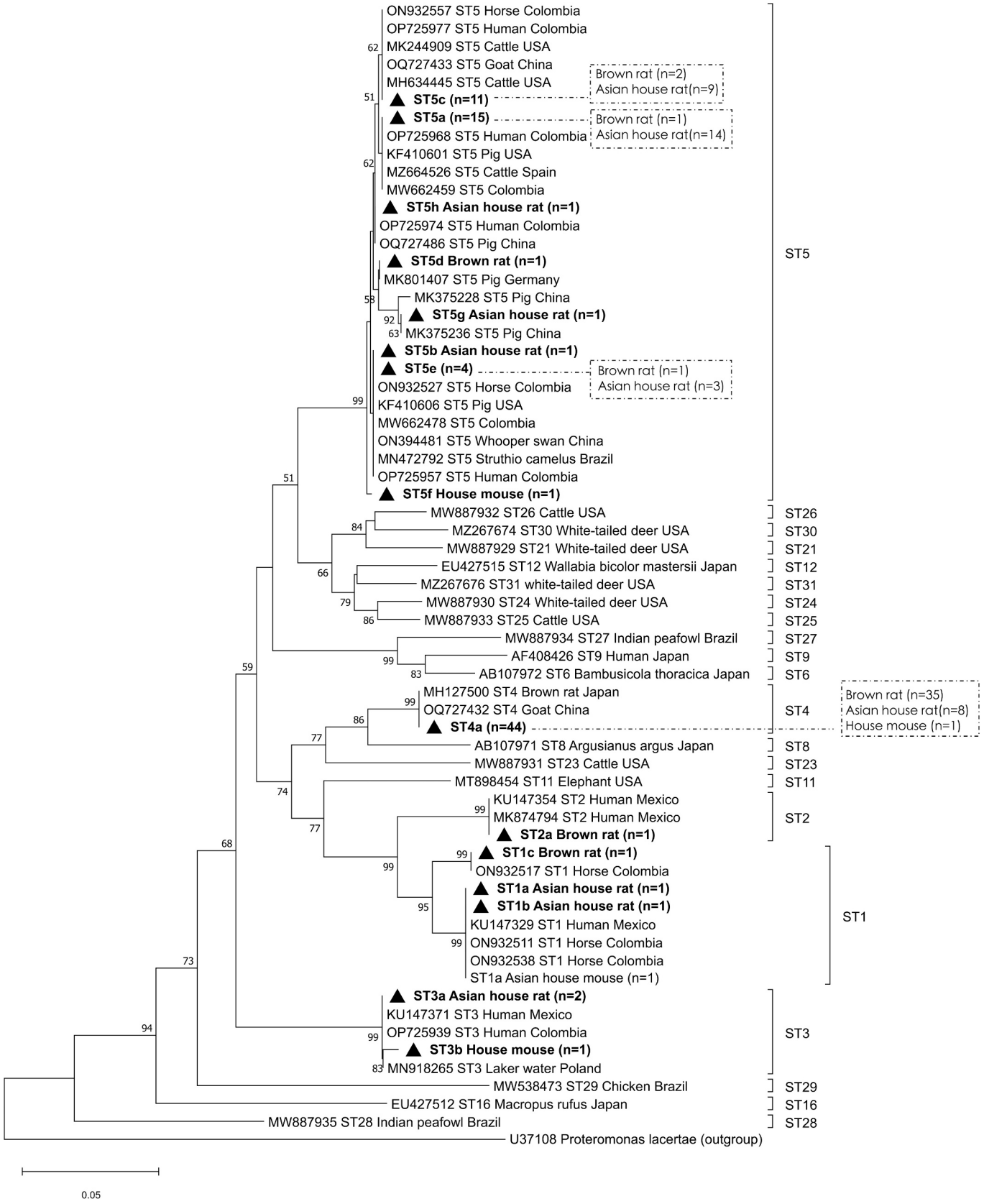

Fig. 3. Phylogenetic relationships of isolates of the *Blastocystis* subtypes from wild small mammals within pig farms. Relationships were analyzed using the neighbor-joining method and the Kimura 2-parameter model based on sequence analysis of the SSU rRNA gene. The black filled triangles in front of the sample names represent the subtypes in the study. Bootstrap values (> 50) are indicated at the nodes. *Proteromonas lacertae* (U37108) was used as the outgroups.

emphasizes the importance of regularly cleaning piggeries to prevent such infections.

Rodent species around the world have reported a total of 13 subtypes (ST1–ST8, ST10, ST13–ST15, and ST17) [12,51]. In this study, we have successfully identified five known zoonotic subtypes (ST1–ST5), with ST4 and ST5 being the first and second most dominant subtypes. However, there are variations in the subtype distribution among the three rodent species in this study. In *R. norvegicus* samples, ST4 emerged as the most predominant subtype, comprising 83.3% (35/42) of the analyzed samples, which is comparable to the prevalence documented in wild brown rats in Malaysia (91.5%, 43/47) and farmed Coypus in China (75.0%, 33/44). In *R. tanezumi* samples, the most dominant subtype was identified as ST5, constituting a substantial proportion of 72.5% (29/40) in the analyzed samples, which significantly exceeds the sporadic occurrence of ST5 observed in rodents around the world [12,51]. As for *M. musculus* samples, a uniform distribution of ST1-ST3 was observed. These differences further underline the varying susceptibility of rodent species to different subtypes of *Blastocystis*.

ST4 infection is the most prevalent subtype among rodent species, reported in >17 rodent species [12,19,26,51]. Our study found that ST4 is not only the most common subtype, but was also detected in all three species of *Blastocystis*-positive rodents, indicating its successful adaptation to infect rodents. It is also one of the four most frequently observed subtypes in the human population. It has been linked to infectious diarrhea in European countries such as Denmark and Spain, posing a significant public health concern [52–54].

Although previous reports had only detected ST5 sporadically in rodents [12,51], our study identified it as the dominant subtype in *R. tanezumi*, with the widest distribution at the pig farm level. Furthermore, a porcine intestinal parasites survey conducted concurrently in pig farms in our sampling area revealed a *Blastocystis* detection rate of 26.2% (368/1402) out of all 1402 pig fecal samples collected. Among these positive samples, the ST5 subtype was found in the majority (91.0%, 335/368), followed by ST1 (4.6%, 17/368) and ST3 (4.3%, 16/368) (unpublished data by Yufeng Liu). These results align with previous studies that have also detected the ST5 subtype as the most prevalent in pigs globally and in some regions of China [12,25,62,63]. Sequence analysis in our study revealed a high homology between the isolated ST5 sequences and GenBank registered sequences identified from pigs (including pigs from the same region as our study by Yufeng Liu) and human samples (Table 3).These findings strongly indicate a cross-species transmission of ST5 among pigs, sympatric rodents, and humans. Moreover, although ST5 is not the predominant subtype of human infection with Blastocystosis, it has been detected in populations of several countries, including China, indicating that sympatric rodents are potential reservoirs at risk of transmitting *Blastocystis* to humans. Public health authorities should be concerned about this potential transmission.

In contrast, ST1–ST3 infections were rarely observed in rodents, with ST1 identified in brown rat populations in Iran and Malaysia [31,42], capybaras in Brazil [46], and flying squirrels and Pallas's squirrels in China [30,34]; ST2 in capybaras in France and black rats in Colombia [37,47]; and ST3 in brown rats in Iran [31], and flying squirrels and Pallas's squirrels in China [30,34] (Table S2). Consistent with these findings, our study revealed a low proportion of ST1 (3.5%, 3/86), ST2 (1.2%, 1/86), and ST3 (3.5%, 3/86) in rodents. However, it is worth noting that >80% of human Blastocystosis is attributed to ST1–ST3 infections, suggesting a high zoonotic threat of these three subtypes [18]. Coupled with recent reports, the ST1–ST5 subtypes have been identified in patients from various provinces of China, including the regions surveyed in our study [55,56,61], emphasizing the concern for zoonotic transmission of rodents as reservoirs and vectors of *Blastocystis*. Therefore, considering the multiple transmission pathways of *Blastocystis*, the risk of infection to humans and other host animals surrounding these farms should not be disregarded [5,19,32].

Rodents are most widely distributed mammals found in natural and human-altered habitats, particularly in agricultural regions [24]. Existing data suggest that rodents can act as natural reservoirs and vectors for *Blastocystis*, implying that endemic infection among these populations can greatly facilitate the transmission of *Blastocystis* in the environment and to other susceptible host species [51,57]. The prevalence of *Blastocystis* infection in reared pigs is remarkably high, with a rate of 52.4% [25]. This indicates that pig farms serve as a significant reservoir for *Blastocystis*. Moreover, rodents present on these farms can potentially act as a risk factor for the spillover transmission of *Blastocystis* from pig farms. To have increased opportunities for accessing food from these sources, on-farm sympatric rodents often inhabit areas near livestock and humans. In times of abundant food on farms, they gather, feed, and reproduce on the premises. This behavior facilitates the circulation of *Blastocystis* through the fecal-oral chain between reared pigs, humans, and rodents. Conversely, as farms are abandoned and food becomes scarce, these rodents embark on migratory journeys searching for suitable habitats. During these journeys, they unintentionally become vectors for the transmission of *Blastocystis* from farms to the wild environment and wildlife.

Evaluating *Blastocystis* zoonotic transmission risk in a specific region heavily relies on the percentage of zoonotic *Blastocystis* subtypes found within animal isolates. This research uncovers a high prevalence of *Blastocystis* infection among sympatric rodents in pig farms, with all isolates identified belonging to zoonotic subtypes. The ST5 subtype, which was previously uncommon in rodents but frequently found in reared pigs, was dominant presence in our study. These findings highlight the significant role of sympatric rodents as reservoirs and vectors in the zoonotic transmission of *Blastocystis* in the investigated areas. Moreover, considering *Blastocystis* has been detected in water and fresh produce [58–60] and that rodents forage in agricultural and vegetable fields and water sources, it raises concerns about rodents as potential environmental sources of infection in the epidemiology of this parasite. Therefore, for a complete understanding of the risk of zoonotic transmission of *Blastocystis* infection, it is crucial to adopt a One Health approach. This entails expanding the sample size to include various types of samples such as humans, livestock, wildlife, water and soil, and extend the geographical coverage of the area under investigation.

5. Conclusions

Blastocystis infection was found to be highly prevalent in free-living sympatric rodents on pig farms in China. Five known zoonotic subtypes (ST1–ST5) were identified. Notably, this investigation extends the host range of *Blastocystis* by documenting the presence of *Blastocystis* infection in *R. tanezumi* and *M. musculus* for the first time. The results suggest that sympatric rodents within pig farms can serve as reservoirs for *Blastocystis* transmission and may act as vectors to play a role in spillover transmission of zoonotic subtypes from pig farms. These findings improve our understanding of the genetic diversity and host range of *Blastocystis* and provide insights into the transmission dynamics of rodents. They also highlight the need for further epidemiological research from a One Health perspective to better understand the potential transmission in the studied areas.

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Ethics statement

This study was carried out under the Chinese Laboratory Animal Administration Act of 1998. The Research Ethics Committee of Henan Agricultural University reviewed and approved the research procedure. Permission was granted to the study team by farm owners or managers to access the farms and collect samples.

Authors contributions

LZ and GZ conceived and designed the experiments. FS, FW, and SC collected the samples. FS, NW, YL and XC performed the experiments and data analyses. This manuscript was written by FS and revised by GZ and LZ. All authors read and approved the final manuscript.

CRediT authorship contribution statement

Fa Shan: Investigation, Writing – original draft. **Fang Wang:** Investigation, Resources. **Shengke Chang:** Investigation, Resources. **Nanhao Wang:** Investigation, Resources. **Yufeng Liu:** Investigation, Resources, Methodology, Writing – review & editing. **Xi Chen:** Investigation, Resources. **Guanghui Zhao:** Methodology, Writing – review & editing. **Longxian Zhang:** Methodology, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no competing interests.

Data availability

All data generated and analyzed during this study are included in the published article. The representative unique nucleotide sequences obtained were submitted to the GenBank database under the accession numbers OR754903-OR754922.

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