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RESEARCH ARTICLE

Molecular detection of zoonotic *Blastocystis* subtypes in urban rats from Pulau Pinang, Malaysia

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ABSTRACT

Blastocystis sp. is a common intestinal protozoan parasite that colonises humans and various animals, with rodents identified as significant reservoir hosts for zoonotic transmission. This study aimed to investigate the prevalence and subtype distribution of *Blastocystis* sp. among wild rats in Pulau Pinang, Malaysia, while evaluating the associations with host demographics and geographic factors. A total of 150 rodent faecal samples from 12 sampling sites across Pulau Pinang, Malaysia, were examined using microscopy, DNA extraction, and PCR amplification of the SSU-rRNA gene. The overall prevalence of *Blastocystis* was 25.3%, with *Rattus norvegicus* exhibiting the highest infection rate (34.5%), followed by *R. rattus* (19.4%), and *Bandicota bengalensis* (16.7%). No infections were reported in *B. indica* (0.0%). Statistical analysis revealed a significant association between *Blastocystis* infection and rodent species (χ^2 = 11.874, df = 3 and p = 0.008), whereas no association was indicated in gender, development stages, and sampling sites. Due to limited funding, sequencing was conducted on ten positive samples that exhibited well-defined bands. Out of ten, three zoonotic subtypes were identified namely, ST1 (allele 4), ST3 (allele 34), and ST4 (allele 94 and 133). The findings of this study emphasise the role of *R. norvegicus* as the major reservoir host in Pulau Pinang, Malaysia and highlight the zoonotic potential of *Blastocystis* in urban settings.

Keywords: Molecular; Prevalence; Protozoan; *Rattus* sp.; Zoonotic.

INTRODUCTION

Blastocystis sp. is a worldwide distributed unicellular intestinal parasite, inhabiting the gastrointestinal tract of humans and a wide range of animals (Wawrzyniak *et al*., 2013), including rodents. Its clinical signs include symptoms resembling the irritable bowel syndrome (IBS), namely, diarrhoea, nausea, fever, vomiting, bloating and abdominal pain (Kumarasamy *et al*., 2023), whereas no obvious clinical signs were reported in infected rodents. Rodents represent approximately 16% of the worldwide prevalence of *Blastocystis*, with variations in rates depending on host species, detection techniques, and geographical location (Farzam *et al*., 2025). They may play a vital role in the distribution of this zoonotic protozoan parasite either as introducers into the environment or as receptors of an infection that is already established in the water.

Molecular studies indicate that *Blastocystis* consists of a collection of genetically different subtypes (STs) instead of being one species. A minimum of 44 STs have been recorded, but only 30 are deemed valid according to the SSU rRNA sequence divergence (Maloney *et al*., 2022). Multiple STs are zoonotic, with ST1–ST4 frequently infecting humans whereas ST1–ST8, ST10, ST13, ST15, and ST17 reported in rodents (Farzam *et al*., 2025; Hatam-Nahavandi *et al*., 2025). ST1–ST3 and ST8 are associated with transmission through water (Barati *et al*., 2022).

In Southeast Asia, the majority of *Blastocystis* studies have concentrated on livestock and poultry, resulting in limited data on rodents, particularly in Malaysia (Rauff-Adedotun *et al*., 2020). The only study on *Blastocystis* in wild rats was conducted previously by Farah Haziqah *et al*. (2018), who investigated *Blastocystis* infections in 293 rodents from urban areas in Perak and Selangor, revealing an overall prevalence of 45.9% in brown rats (*Rattus norvegicus*). Based on the molecular analysis of 47 isolates, four subtypes were identified, ST4 being predominant (91.5%), followed by ST1 (4.3%), ST5 (2.1%), and ST7 (2.1%). The findings highlight urban rodents, particularly brown rats, as significant reservoirs of *Blastocystis*, with ST4 potentially playing a major role in zoonotic transmission.

Given the widespread occurrence of *Blastocystis* in developing countries and its potential impact on public health, there is a growing need to investigate its distribution and genetic diversity in various animal reservoirs. Rodents are of concern due to their close association with human settlements, their adaptability to diverse environments, and their role as carriers of numerous zoonotic pathogens. In Pulau Pinang, rodent infestation is notably high, especially in densely populated urban areas, wet markets, food premises, and waste disposal sites where food and shelter are abundant (Wan Nur Amni *et al*., 2019). These environments create ideal conditions for rodents to thrive and increase the likelihood of environmental contamination through their urine and faeces. Consequently, the potential for *Blastocystis* transmission

from rodents to humans is amplified, making it crucial to conduct targeted surveillance and genetic characterisation of the parasite in local rodent populations to assess the zoonotic risk and inform appropriate public health interventions. Therefore, this study focuses on examining the occurrence and genetic variation of *Blastocystis* in rodent populations in Pulau Pinang, to provide insights into its potential for zoonotic transmission and to fill existing gaps in local epidemiological data.

MATERIALS AND METHODS

Ethical approval

The wild rats were obtained through a campaign initiated by the Zero Rodent (ZoRo) Project: Rat to Cash 2023 campaign by the Seberang Perai City Council (MPSP) and were euthanised by MPSP staff. As only dead specimens were collected, no permit or ethical approval was required for this study, as confirmed by the Universiti Sains Malaysia Institutional Animal Care and Use Committee (USM IACUC).

Positive *Blastocystis* isolates from wild rats

A total of 150 intestinal samples were collected from wild rats captured across 12 locations in Pulau Pinang, Malaysia (6.5508°N, 22.4568°E), including Air Itam, Balik Pulau, Batu Ferringhi, Bayan Lepas, Bukit Mertajam, Butterworth, Gelugor, Georgetown, Nibong Tebal, Seberang Perai, Simpang Ampat, and Tanjung Tokong. Species, gender, and development stages of rodents were identified based on morphological features Lim (2015). Following *in-vitro* cultivation in Jones' medium, a total of 38 microscopically examined *Blastocystis*-positive isolates were stored at -20°C before DNA extraction.

DNA barcoding method

The genomic DNA of 38 Blastocystis-positive isolates were extracted using Nucleospin® DNA Stool Kit (Macherey-Nagel, Germany), following the manufacturer's protocol (Genomic DNA From Stool Samples, User Manual of NucleoSpin DNA Stool, 2016). Conventional PCR was performed to target a 600 bp fragment of SSU-rRNA gene using the primers RD5 (ATCTGGTTGATCCTGCCAGT) and BhRDr (GAGCTTTTTAACTGCAACAACG) as previously described (Mohd Zain *et al*., 2017). The PCR was conducted by amplifying 2 µL of each of 38 DNA templates in a final volume of 50 µL, composing of 25 µL 2X Taq Master Mix (Vivantis, Czechia), 1 µL of 2.5nM MgCl2 (Vivantis, Czechia), 0.5 µL of each primer (Integrated DNA Technologies, USA), and 21 µL nuclease-free water (Vivantis, Czechia). The PCR was performed with the following optimised conditions: an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 56.3°C for 1 minute 30 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes.

Gel electrophoresis

Approximately 2 µL of each PCR product was resuspended with 2 µL of Bioline 5X DNA Loading Buffer Blue (Meridian Bioscience, USA) on parafilm and was then loaded into the designated wells, along with positive and negative controls. Gel electrophoresis was conducted using MP-300V Power supply (Major Science, USA) set to 100 V for 40 minutes, allowing DNA fragments to migrate from the negative to the positive electrode. DNA bands were visualised using GeneFlash Gel Imaging (Syngene, India). White light and manual CCD camera focus were used to adjust image clarity, and UV light was applied for DNA bands visualisation. The gel images were then saved to a USB drive for subsequent analysis.

DNA Sequencing & Sequence Analysis

Gel electrophoresis images were analysed to confirm the presence of *Blastocystis* in the samples. PCR products showing a distinct band at approximately 600 bp were indicated as positive *Blastocystis*. Among these, ten samples displaying the most intense band signal were selected for sequencing because band intensity reflects DNA concentration and quality, thus increasing the likelihood of successful sequencing. The unpurified PCR amplicons were then sent to the Centre for Chemical Biology (CCB), USM, for reverse direction sequencing.

To determine the *Blastocystis* subtypes, the obtained sequence data were first compared with the sequences available in the database of PUBMLST (https://pubmlst.org/organisms/Blastocystis-spp). Sequences that did not match a defined allele were further analysed using the BLAST database (https://blast.ncbi.nlm.nih.gov/Blast), to determine the closest allele based on sequence similarities (98-100%).

Statistical analysis

Statistical analysis was performed using IBM SPSS version 28 (SPSS, USA). Descriptive analysis was conducted to summarise the demographic characteristics of rodents, including species, gender and development stages across different sampling sites. Percentages (%) were used to describe the prevalence of *Blastocystis* in the rodent population. The Pearson Chi-Square Test (χ^2) was employed to assess the associations between Blastocystis infection status (positive or negative) and host demographic factors (species, gender, development stages and sampling locations) with a significant value of $p < 0.05$.

RESULTS

Prevalence of *Blastocystis* in wild rats

Out of 150 rodents examined, it was found that 38 individuals tested positive for *Blastocystis*, giving an overall prevalence of 25.3%. A significant difference in infection prevalence was observed among rodent species ($\chi^2 = 11.874$, df = 3 and $p = 0.008$). *Rattus norvegicus* (34.5%) recorded the highest prevalence, followed by *R. rattus* (19.4%) and *B. bengalensis* (16.7%), while no infections were reported in *B. indica* (Table 1).

Table 1. Prevalence of *Blastocystis* infection in wild rats in Pulau Pinang, Malaysia

Variable	Infection status (%)		Total (%)
	Negative	Positive	
Species		$p = 0.008$	
R. norvegicus	57 (65.5)	30 (34.5)	87 (58.0)
R. rattus	29 (80.6)	7 (19.4)	36 (24.0)
B. indica	21 (100.0)	0 (0.0)	21 (14.0)
B. bengalensis	5 (83.3)	1 (16.7)	6 (4.0)
Sex		$p = 0.150$	
Male	44 (81.5)	10 (18.5)	54 (36.0)
Female	68 (70.8)	28 (29.2)	96 (64.0)
Development stages		$p = 0.928$	
Juvenile	17 (73.9)	6 (26.1)	23 (15.3)
Adult	95 (74.8)	32 (25.2)	127 (84.7)
Sampling sites		$p < 0.001$	
Air Itam	3 (100.0)	0 (0.0)	3 (2.0)
Balik Pulau	11 (100.0)	0 (0.0)	11 (7.3)
Batu Ferringhi	14 (93.3)	1 (6.7)	15 (10.0)
Bayan Lepas	7 (77.8)	2 (22.2)	9 (6.0)
Bukit Mertajam	12 (100.0)	0 (0.0)	12 (8.0)
Butterworth	28 (50.0)	28 (50.0)	56 (37.3)
Gelugor	5 (50.0)	5 (50.0)	10 (6.7)
Georgetown	5 (71.4)	2 (28.6)	7 (4.7)
Nibong Tebal	2 (100.0)	0 (0.0)	2 (1.3)
Seberang Perai	19 (100.0)	0 (0.0)	19 (12.7)
Simpang Ampat	3 (100.0)	0 (0.0)	3 (2.0)
Tanjung Tokong	3 (100.0)	0 (0.0)	3 (2.0)
Total no. of rodents (%)	112 (74.4)	38 (25.3)	150

*Significance value ($p < 0.05$) was calculated based on the difference between variables among all rodents using the Pearson Chi-Square Test.

Based on sex, female rodents exhibited a higher prevalence of *Blastocystis* infection (29.2%) compared to males (18.5%), while juveniles (26.1%) showed a slightly higher prevalence than adults (25.2%). However, these differences were not statistically significant for either developmental stage (χ^2 = 0.008, df = 1, p = 0.928) or sex (χ^2 = 2.072, df = 1, p = 0.150) (Table 1).

Among all 12 sampling sites, no *Blastocystis* infection was detected in rodent populations from Air Itam, Balik Pulau, Bukit Mertajam, Nibong Tebal, Seberang Perai, Simpang Ampat, and Tanjung Tokong. In contrast, infections were observed in five locations, with Butterworth and Gelugor showing the highest *Blastocystis* prevalence in rodents (50.0%), followed by Georgetown (28.6%), Bayan Lepas (22.2%), and Batu Ferringhi (6.7%). A significant association was revealed statistically between *Blastocystis* infection and sampling sites of rodents (χ^2 = 42.740, df = 12 and p < 0.001) (Table 1).

***Blastocystis* subtype distribution in wild rats**

A total of 10 *Blastocystis*-positive samples from *R. norvegicus* and *R. rattus* that showed the most intense band signal in gel electrophoresis were selected for sequencing. Most of these samples were obtained from adult female rodents, primarily collected from Butterworth. There were seven *R. norvegicus* samples were successfully classified into three zoonotic subtypes, while the remaining samples failed to match with the database in PUBMLST. The identified subtypes were ST1 (allele 4) (n = 3), ST3 (allele 34) (n = 2), and ST4 (allele 94 and 133) (n = 2) (Table 2).

DISCUSSION

The overall prevalence of *Blastocystis* in wild rats from Pulau Pinang was relatively low compared with the previous findings by Farah Haziqah *et al*. (2018), who conducted the study on wild rats in the central region of Peninsular Malaysia, who reported a very high prevalence of *Blastocystis* infection with 45.9% in wild rats from Perak and Kuala Lumpur.

Among the species, *R. norvegicus* showed the highest prevalence and was identified as the main reservoir host, consistent with previous studies (Wang *et al*., 2018b; Bastaminejad *et al*., 2024; Gao *et al*., 2024). These differences suggested that it could be due to the variations in ecological behaviour of rodents, including dietary habits and habitat preferences, as frequent exposure to contaminated environments or food sources might influence the infection rates, further increasing host interaction with the pathogen (Bordes *et al*., 2013). *Rattus norvegicus* and *R. rattus* were highly commensal rodent species that lived close to human settlements (Bastaminejad *et al*., 2024; Shan *et al*., 2024), with *R. norvegicus* also known as Norway or brown rat served as an omnivorous generalist that fed on a wide range of food, including human waste, thereby contributed a higher exposure to contaminated environments (Klemann & Pelz, 2005) whereas, *R. rattus* also known as roof or black rat were mostly herbivores with a stronger dietary preference for fruits, seeds and plants, which preferred to occupy elevated regions such as ceilings, attics or trees, thus less likely to contact to ground-level contaminants (Nascimento *et al*., 2019). *B. bengalensis* (lesser bandicoot rat) and *B. indica* (greater bandicoot rat) were classified as field rats, typically associated with rural agricultural settings in which their primary diet mainly relied on stored grains (Rosentrater, 2022). Besides, their habitat preferences generally limit the interaction with contaminated urban waste or water sources, while keeping a distance from human habitation.

Although female rodents exhibited higher *Blastocystis* infection rates compared to males, the infection rates were not likely to be affected by gender, which aligned with the findings from Shan *et al*. (2024). However, there was no significant sex-related effect observed for *Blastocystis* infection in rodents (Gao *et al*., 2024; Shan *et al*., 2024), as both sexes inhabited a similar ecological niche which had comparable exposure to contaminated environments, leading to an equal risk of infections. As noted by Schmid-Hempel (2021), sex-based differences in immune response could also influence the susceptibility to parasitic infections, but it might not be strongly expressed in *Blastocystis* infection. Correspondingly, infection rates were also not found to be influenced by the development stages of the rodent (Shan *et al*., 2024). Although juveniles had a slightly higher *Blastocystis* prevalence than adults, the difference was not statistically significant. Juveniles were generally more vulnerable to parasitic infections due to their immature immune systems and more exploratory behaviour, increasing the likelihood of encountering contaminated substances. Since both juveniles and adults often share the same foraging and nesting environments, they are likely to have similar exposure to potential sources of infection.

The higher number of *Blastocystis*-positive rodents in Batu Ferringhi, Bayan Lepas, Butterworth, Gelugor and Georgetown might be attributed to high population density combined with poor waste management, which provided abundant food sources and nesting sites for rodents, thereby increasing their exposure to pathogens

Table 2. Subtypes of *Blastocystis* from wild rats in Pulau Pinang

Species	Field No.	Gender	Development stage	Sampling site	Subtype (allele)
R. rattus	TSN1	Male	Adult	Bayan Lepas	NA
R. norvegicus	PMM18	Female	Juvenile	Butterworth	NA
	PMM9	Female	Adult	Butterworth	NA
	PMM30	Male	Adult	Butterworth	ST1 (4)
	PMM20	Female	Adult	Butterworth	
	PMM22	Female	Adult	Butterworth	
	PMM15	Female	Adult	Butterworth	ST3 (34)
	ATS3	Female	Adult	Georgetown	
	PMM29	Female	Adult	Butterworth	ST4 (94)
	BD1	Female	Adult	Bayan Lepas	ST4 (133)

NA = Sequence did not match any defined allele or subtype in the PUBMLST database.

(Zubair *et al*., 2024). Improper sewage system, particularly the presence of open drainage channels in housing areas, markets and food courts, potentially created environmental hotspots for *Blastocystis* transmission among rodents and facilitated zoonotic transmission to humans (Matovelle *et al*., 2022).

Previous studies have identified a wide range of *Blastocystis* subtypes (STs) circulating in rodent populations worldwide. To date, there were 12 subtypes (ST1–ST8, ST10, ST13, ST15, and ST17) reported (Cian *et al*., 2017; Farah Haziqah *et al*., 2018; Wang *et al*., 2018a,b; Betts *et al*., 2018; Xiao *et al*., 2019; Mohammadpour *et al*., 2020; Martínez-Hernández *et al*., 2020; Liu *et al*., 2024; Farzam *et al*., 2025) in which rodents can harbour both zoonotic subtypes commonly found in humans (such as ST1–ST4) as well as host-adapted subtypes that are less frequently detected in other animals. The diversity of these protozoan subtypes among rodents suggests their important role as potential reservoirs for zoonotic transmission, particularly in urban and peri-urban environments where human–rodent interactions are frequent.

ST1 is the most common subtype due to its wide host range and frequent occurrence in both humans and a wide range of animals, including rodents (Cui *et al*., 2025; Farzam *et al*., 2025; Hatam-Nahavandi *et al*., 2025). Experimental evidence has shown that ST1 can colonise rodents without harmful effects (Skotarczak, 2018) and has been reported previously in wild rats from Peninsular Malaysia by Farah Haziqah *et al*. (2018). Not only in humans and animals, but this subtype has also been detected in diverse water types (Attah *et al*., 2023). Although ST1 is recognised as a zoonotic subtype found in animals and humans, allele level information in animal hosts is scarce. These ST1 (allele 4) isolates are most represented in humans, as reported in several studies (Ahmed *et al*., 2022; Aykur *et al*., 2023; Marangi *et al*., 2023). However, this study successfully identified ST1 (allele 4) in *R. norvegicus* caught in a major town in the state of Pulau Pinang, highlighting the potential role of this highly adaptable urban pest as reservoirs for zoonotic *Blastocystis* subtypes.

ST3 was less commonly encountered in the rodent population, but it was detected in this study and previously reported in *R. norvegicus* from Iran (Mohammadpour *et al*., 2020). As a zoonotic subtype, it potentially originated from human hosts, specifically among the immunocompromised patients (Mohammad Rahimi *et al*., 2022). Lately, it has been recognised as the dominant subtype in Malaysian human populations (Rozani *et al*., 2025). The presence of *Blastocystis* ST3 in the urban rodent population was not unexpected, as this pest lives near humans and frequently encounters human waste, sewage, garbage, and contaminated water sources. This study found for the first time the occurrence of *Blastocystis* ST3 (allele 34) in *R. norvegicus* in Malaysia. These alleles are among the most frequently reported alleles in humans, domestic animals, namely, dogs and cats, as well as the synanthropic animals commonly found in brown rats (Mohammadpour *et al*., 2020).

ST4 was well-documented as a rodent-associated subtype (Cian *et al*., 2017; Farah Haziqah *et al*., 2018; Wang *et al*., 2018b; Gao *et al*., 2024). It had been consistently found in *R. norvegicus* across diverse regions, including France (Cian *et al*., 2017), China (Wang *et al*., 2018b; Gao *et al*., 2024; Liu *et al*., 2024; Shan *et al*., 2024; Wang *et al*., 2024), and Iran (Mohammadpour *et al*., 2020; Bastaminejad *et al*., 2024). As noted by Farah Haziqah *et al*. (2018), ST4 was confirmed as the most prevalent subtype in the Malaysian rat population. Hence, the detection of ST4 in this study supported the hypothesis that *R. norvegicus* potentially served as a natural reservoir host for ST4 and other zoonotic subtypes in Pulau Pinang, Malaysia. There were two ST4 alleles found in *R. norvegicus* (94 and 133), both has been identified in humans (Stensvold *et al*., 2012; Paulos *et al*., 2018). ST4 (allele 94) was previously found in dogs as reported by Mohammadpour *et al*. (2020) and Marangi *et al*. (2023). Meanwhile, ST4 (allele 133) has been specifically identified in a non-human primate (NHP), a single woolly monkey from a zoological garden in the UK (Alfellani *et al*., 2013).

However, there were three samples isolated from *Rattus rattus* was unidentified, and this could possibly be due to genetic variations that differ from the currently defined alleles and subtypes in PUBMLST. To our knowledge, this is the first study to report allele-level characterisation of *Blastocystis* from rats (*Rattus* spp.) (Figure 1). Thus, the findings from this study indicate possible spillover at the human–rodent interface.

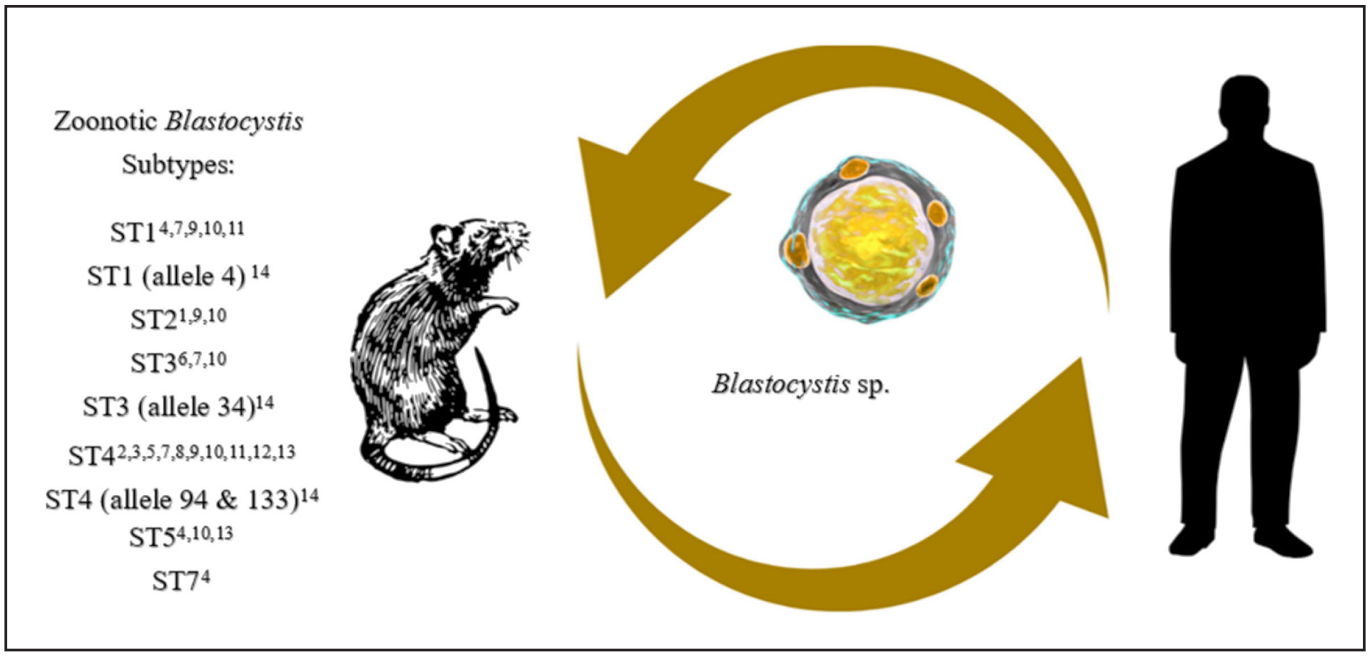

Figure 1. *Blastocystis* subtypes and alleles isolated from rats (*Rattus* spp.) worldwide. [1]Ramírez *et al*. (2013), [2]Yoshikawa *et al*. (2016), [3]Cian *et al*. (2017), [4]Farah Haziqah *et al*. (2018), [5]Wang *et al*. (2018b), [6]Valenna-Barbosa *et al*. (2019), [7]Mohammadpour *et al*. (2020), [8]Tantrawatpan *et al*. (2023), [9]Liu *et al*. (2024), [10]Shan *et al*. (2024), [11]Bastaminejad *et al*. (2024), [12]Wang *et al*. (2024), [13]Gao *et al*. (2024), [14]Present study.

CONCLUSION

This study highlights the importance of continuous molecular surveillance of *Blastocystis* in wild rat populations to better understand their role in zoonotic transmission and to support public health measures aimed at reducing the risk of parasite spread in urban environments. Additionally, the detection of three zoonotic subtypes (ST1, ST3, and ST4) identified in this study underscores the potential risk of cross-species transmission between rodents and humans, particularly in urban areas where close contact may occur.

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Conflict of interest

The authors declare no competing interests.

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