Short Communication

Re-survey of Blastocystis infection in free-living birds from urban Xinxiang city

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Abstract

Blastocystis is the most ubiquitous intestinal protozoan parasite found in humans and animals worldwide. Commonly, ST7 and ST6 are the major *Blastocystis* subtypes reported in birds, and their frequencies fluctuate greatly with various factors. However, little literature is available on the prevalence and subtype characteristics of this parasite in free-living wild birds in the same city. For this purpose, a total of 114 fresh fecal specimens were collected from wild free-living birds in urban districts of Xinxiang city and tested for *Blastocystis* using PCR of the barcoding region of the small subunit ribosomal RNA (SSU rRNA) gene. The positive isolates were subsequently subtyped by sequence analysis, and *Blastocystis* was detected in 19 of the fecal specimens, with a prevalence of 16.7% (19/114), and only ST7 was identified. As a zoonotic subtype, the potential transmission threat of *Blastocystis* to humans could not be ignored. Interestingly, the findings in the current study were significantly different from the lower prevalence and inconsistent subtypes detected in previous studies, suggesting the incidence of *Blastocystis* ST7 in birds depends on multiple factors, while simultaneously exposing its non-pathogenic host adaptability.

Keywords: *Blastocystis*, free-living wild birds, prevalence, subtype, urban Xinxiang

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Introduction

Blastocystis, a unicellular anaerobic microbial eukaryote, is one of the most common protists in the gastrointestinal tract of a wide range of hosts worldwide, including mammals, birds, and reptiles (Andersen and Stensvold, 2016; Kwon *et al.*, 2024). Currently, the clinical significance of *Blastocystis* remains debated, because the infections can be both asymptomatic and symptomatic in humans (Wakid *et al.*, 2022; Aykur *et al.*, 2024; Wei *et al.*, 2024). Some symptoms, such as diarrhea, nausea, irritable bowel syndrome, rash, gas, and urticaria, are considered to be associated with this protozoan (Ajjampur and Tan, 2016; Figueiredo *et al.*, 2024). However, *Blastocystis* infections are more frequently reported in non-diarrheic individuals globally, including domestic and wild animals (Hublin *et al.*, 2021; Guilavogui *et al.*, 2022; Liu *et al.*, 2022a; Liu *et al.*, 2022b). Recent studies indicate that *Blastocystis* is increasingly viewed as a commensal member of the gut microbiome, as its colonization promotes a healthier gut and microbiome by increasing the diversity of beneficial bacteria (Deng *et al.*, 2021a; Feranmi, 2022; Aykur *et al.*, 2024). In addition, some surveys confirm high frequencies (100% prevalence) in hosts without any clinical signs, supporting the thought that *Blastocystis* is a biomarker organism or potential modulator of a healthy gut microbiota (Jinatham *et al.*, 2021; Vasana *et al.*, 2021; Stensvold *et al.*, 2022; Santin *et al.*, 2023). The transmission of *Blastocystis* occurs primarily through the fecal-oral route, including the ingestion of contaminated water or food, and direct contact with contaminated environments and infected hosts (Jinatham *et al.*, 2021; Figueiredo *et al.*, 2024; Kwon *et al.*, 2024).

Blastocystis exhibits extensive genetic variation in different hosts and regions, and based on polymorphisms in the small subunit rRNA gene (SSU rRNA), at least 46 subtypes (STs) are currently recognized (Aykur *et al.*, 2024; Figueiredo *et al.*, 2024; Koehler *et al.*, 2024). Of these, fifteen STs, including ST1-10, ST12, ST14, ST16, ST23, and ST24, have been reported in both humans and animals at varying prevalences, indicating zoonotic potential (Maloney *et al.*, 2022; Li *et al.*, 2023). Given molecular studies, ST1-ST4 represents more than 90% of human-derived *Blastocystis* isolates (Deng *et al.*, 2019a). In addition, host-adaptation can be seen, such as ST5 in pigs, ST7 and ST6 in birds, as well as ST10, ST14, ST24-ST26, and ST42-ST44 in ruminants (Hublin *et al.*, 2021; Rauff-Adedotun *et al.*, 2021; Aykur *et al.*, 2024). Additionally, several STs can concurrently colonize one host (Maloney *et al.*, 2021; Koehler *et al.*, 2024).

Many studies on the molecular epidemiology of *Blastocystis* infection have been conducted in captive birds, including wild, pet, and poultry, where ST7 and ST6 were identified as the predominant subtypes (Liu *et al.*, 2022a; Li *et al.*, 2023; Sanggari *et al.*, 2023). However, most of these studies focus on cross-sectional surveys for specific animal species or breeding areas. To our knowledge, data on the longitudinal tracing of *Blastocystis* infection in free-living wild birds in urban areas are rare. Therefore, the primary objective of this study is to determine the prevalence and subtype variation of *Blastocystis* in free-living wild urban birds in the warmer seasons, to assess the zoonotic health risk.

Materials and Methods

Ethical approval: Ethical approval for specimen collection was reviewed and obtained from the Ethics Committee of the Henan Institute of Science and Technology (LLSC2024030).

Specimen collection: From March to September 2023, a total of 114 fresh fecal specimens were collected from free-living wild birds in an urban district of Xinxiang City (N35°18′, E113°54′), China, covering two universities and two communities. All the feces were obtained from the ground after the natural defecation of birds in the morning. The sampling protocol was consistent with that of a previous survey (Li *et al.*, 2023). All specimens collected in this study were considered healthy based on the normal shape of the feces, and samples were sent to the laboratory immediately under cold conditions for DNA extraction.

DNA extraction: Genomic DNA was extracted from approximately 200 mg of each fecal sample using the E.Z.N.A. Stool DNA kit (Omega, Norcross, GA, USA), following the manufacturer's instructions. The DNA was eluted in 200 μL of the provided elution buffer and subsequently stored at -20°C for PCR analysis.

PCR amplification: All samples were tested for *Blastocystis* presence by PCR amplification of a ~600 bp fragment of the SSU rRNA gene of the barcoding region. The primers BhRDr (5′-GAGCTTTTTAACTGCAACAACG-3′) and RD5 (5′-ATCTGGTTGATCCTGCCAGT-3′) were used and PCR reaction conditions were as follows: 94°C for 4 min; 35 cycles of 94°C for 45 s, 59°C for 45 s and 72°C for 1 min; and 72°C for 5 min for a final extension (Scicluna *et al.*, 2006; Liu *et al.*, 2021b). A Taq PCR MasterMix was used for all reactions, and each DNA specimen was subjected to two repetitions. Both positive (ST7 from quail) and negative (ultrapure H_2O) controls were used to detect contamination in each PCR reaction. The amplified products were evaluated by 1% agarose gel electrophoresis with GelRed staining (Solarbio, China).

DNA sequencing analysis: All positive PCR products were sequenced at Sangon Biotech Co., Ltd (Shanghai, China) with bidirectional sequencing to ensure nucleotide accuracy. The obtained sequences were aligned with each other using ClustalX v2.1 (http://www.clustal.org/) and any inconsistent sequences were subjected to BLAST analysis (https://blast.ncbi.nlm.nih. gov/Blast.cgi) to determine the *Blastocystis* subtype.

Phylogenetic analysis: A neighbor-joining (NJ) phylogenetic tree was constructed using Mega 7.0 (https://www.megasoftware.net/) for all *Blastocystis* sequences to further identify subtypes. Reference sequences of the SSU rRNA gene were downloaded

from the GenBank database and previously reported literature. The Kimura 2-parameter model and bootstrap analysis of 1,000 replicates were used to evaluate the reliability and statistical support of the produced topology and to compute evolutionary distances. Only branch support values above 50% are shown.

Nucleotide sequence accession numbers: The representative nucleotide sequence of all the *Blastocystis* ST7 sequences obtained in this study was deposited in the GenBank database under accession number: PV665019.

Result and Discussion

Of the 114 fecal specimens obtained from free-living wild birds, 19 tested positive for *Blastocystis* via PCR, with a final prevalence of 16.7%. In detail, 33.3% (7/21) was found on the grounds of University 1, with a 12.7% (9/71) prevalence in University 2 and a 50% prevalence in the Community 2 subsample. No infection was detected in Community 1 (Table 1). Among the three positive sampling sites, differences in the infection rates were not significant. This study constitutes the second report to Xinxiang City, and the current infection rate was notably higher compared to the previous results (1.5%, 2/136) (Li *et al.*, 2023). For birds (domestic and wild), the prevalence of *Blastocystis* varied largely in other studies, ranging from 0 to 100% (Deng *et al.*, 2019b; Deng *et al.*, 2021b; Liu *et al.*, 2021b; Liu *et al.*, 2022a; Naguib *et al.*, 2022; Guyard-Nicodeme *et al.*, 2023; Tantrawatpan *et al.*, 2023; Zhang *et al.*, 2023; Feng *et al.*, 2024; Wang *et al.*, 2024; Xue *et al.*, 2024). This large variation can only be explained by the interaction of multiple factors, including sanitation, management, population density, living conditions, and feeding mode (Deng *et al.*, 2021b; Barati *et al.*, 2022; Liu *et al.*, 2022a; Su *et al.*, 2022). Additionally, detection method, instrumentation, reagents, and primers are also important factors affecting the detected *Blastocystis* prevalence in various hosts (Maloney *et al.*, 2020; Liu *et al.*, 2021b; Su *et al.*, 2022; Guyard-Nicodeme *et al.*, 2023; Li *et al.*, 2023; Zhang *et al.*, 2023). In this study, the overall prevalence (16.7%) was similar to that seen in the red-crowned crane (14%, 6/43) in a reserve in Heilongjiang province and green peafowl (17.7%, 3/17) from a zoo in Henan province (Wang *et al.*, 2018; Zhang *et al.*, 2021), but was lower than the prevalence detected in ostrich studies with smaller sample sizes (31.6% to 42.9%) (Zhao *et al.*, 2018; Deng *et al.*, 2021b; Zhang *et al.*, 2021). However, primers, PCR methods, collection time, and reagents from different manufacturers are likely to be mainly responsible for the current different results (Li *et al.*, 2023). Furthermore, these factors possibly also contribute to the variable infection rates in various bird species from different regions.

Sequence analysis revealed that all the positive isolates belonged to the ST7 subtype (Fig. 1). Among them, identical nucleotide sequences were found, with 100% similarity to ST7 found in quails and only 1-2 nucleotide differences to sequences found in peafowls from previous studies in the surrounding area (Liu *et al.*, 2021a; Liu *et al.*, 2021b). In view of recent epidemiological surveys, ST7 was the dominant subtype found in domestic and wild birds, followed by ST6 (Barati *et al.*, 2022; Naguib *et al.*, 2022; Tantrawatpan *et al.*, 2023; Zhang *et al.*, 2023; Feng *et al.*, 2024; Wang *et al.*, 2024; Xue *et al.*, 2024). In addition, several STs (ST1, ST4, ST5, ST8, ST8, ST10, ST14, ST23, ST24, and ST25), containing potentially novel STs, had been reported in a few avian hosts (Su *et al.*, 2022; Li *et al.*, 2023; Tantrawatpan *et al.*, 2023; Zhang *et al.*, 2023 Feng *et al.*, 2024; Xue *et al.*, 2024). Thus, *Blastocystis* ST7 seems to be a dominant avian-adapted subtype. However, many genetic polymorphisms were confirmed between the intra-subtypes of ST7 from the same bird species or among different avian hosts (Liu *et al.*, 2021b; Liu *et al.*, 2022a; Aykur *et al.*, 2024; Feng *et al.*, 2024). The reason for this remains unclear, but it might be that the SSU rRNA gene is not adequate for the identification and classification of ST7-type *Blastocystis* or a stronger host-adaptation may be present here (Rezaei Riabi *et al.*, 2018; Aykur *et al.*, 2024; Yuan *et al.*, 2024). In the present study, the intra-genetic homogeneity of ST7 might indicate that these birds did not frequently move to other habitats or regions. In addition, previous surveys sampled during cold climates, while this experiment was conducted during warmer climates, which might explain the high incidence of ST7.

Previously, *Blastocystis* ST7 was defined as a pathogenic parasite, due to the presence of diarrhea in humans and mice (Yason *et al.*, 2019; Deng *et al.*, 2022a). However, with the discovery and reporting of a large number of ST7 isolates, most animal hosts colonized by this subtype showed no clinical symptoms, especially not wild birds (Barati *et al.*, 2022; Zhang *et al.*, 2023; Xue *et al.*, 2024). Thus, the ST7 isolates commonly found in animals could be a non-pathogenic strain. Remarkably, *Blastocystis* is most commonly reported in studies of asymptomatic individuals, and increasing evidence suggests that *Blastocystis* belongs to a beneficial intestinal symbiosis and can be considered a biomarker or an indicator of a healthy gut (Billy *et al.*, 2021; Deng *et al.*, 2021a; Deng *et al.*, 2022b; Feranmi, 2022; Stensvold *et al.*, 2022; Deng *et al.*, 2023). This notion is supported by studies on asymptomatic chickens, ducks, and geese (Falkowski *et al.*, 2022; Liu *et al.*, 2022a; Gomes-Gonçalves *et al.*, 2024). Therefore, the detection of ST7 from avian hosts probably indicates a healthy or normal bird. Besides, all the fecal specimens from this study appeared normal, similar to the samples from the above-mentioned studies. Unfortunately, studies on the cross-transmission of this type of ST7 to humans are scant, and the transmission risk to residents should not be ignored in this city.

This study is the second survey of the infection state of *Blastocystis* in free-living birds in urban Xinxiang city. A higher infection rate and obvious subtype divergence were observed compared to the first survey. Stronger host-adaptation was seen for the ST7 strain in free-living birds, but the potential zoonotic risk remains, and health authorities need to remain vigilant. In addition, routine surveillance of urban wildlife should be conducted to mitigate public health implications for urban residents.

Table 1 Prevalence and distribution of Blastocystis subtypes in wild free-living birds in the present study.

Location/ sampling site	No. specimens	No. positive	Prevalence	*P*	Subtype
University 1	21	7	33.3%	0.028	ST7 (7)
University 2	71	9	12.7%	reference	ST7 (9)
Community 1	16	0	0	0.133	
Community 2	6	3	50%	0.016	ST7 (3)
Total	114	19	16.7%		ST7(19)

Reference: a baseline group

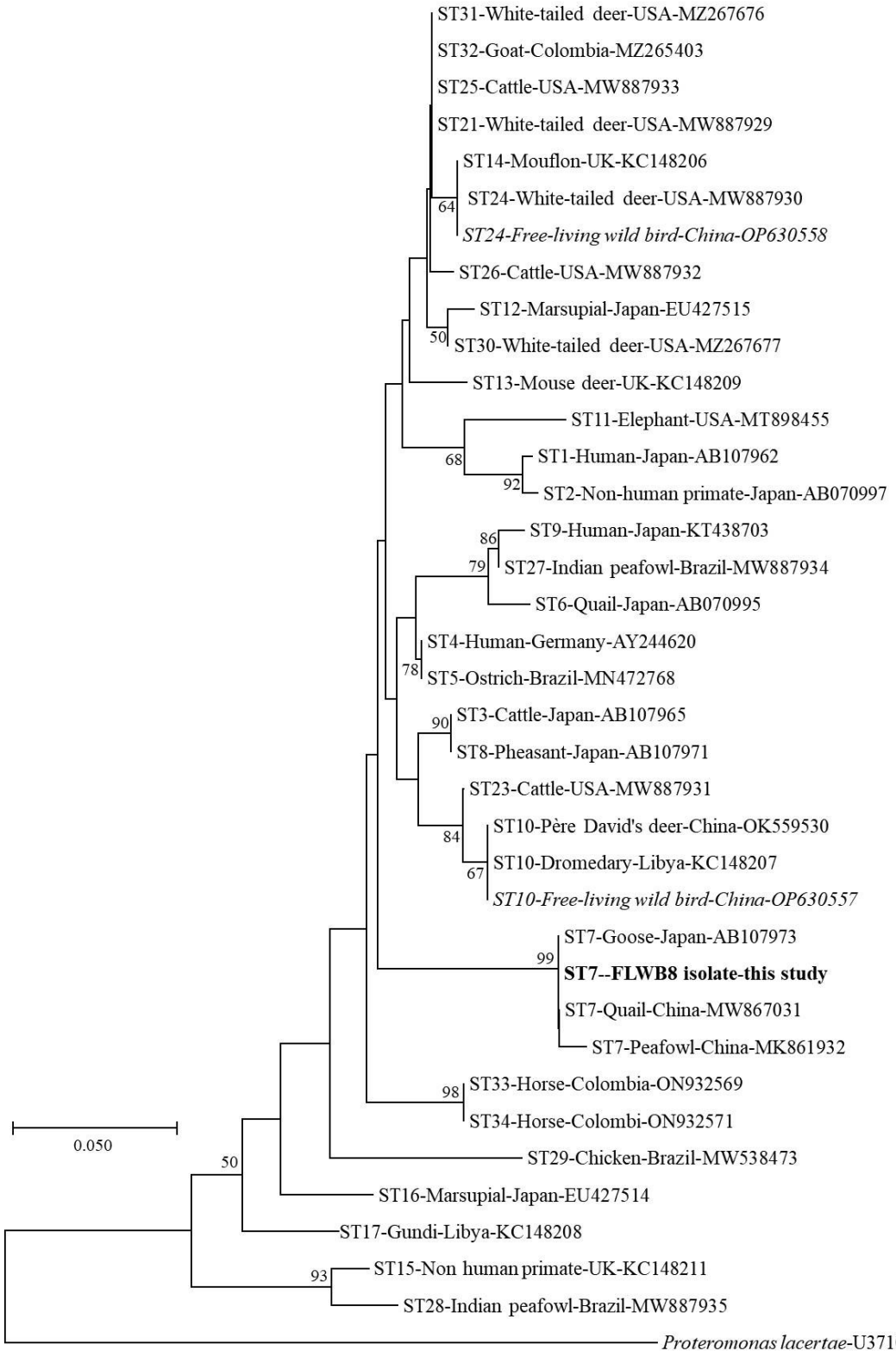

Figure 1 Neighbor-joining (NJ) tree of the SSU rDNA gene of Blastocystis isolates from free-living wild birds in urban China in relation to reference sequences. Bootstrap values were obtained using 1,000 pseudoreplicates, and those with >50% are shown. Proteromonas lacertae was used as the outgroup. Bar = substitutions/site.

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Availability of data and materials: All the sequences obtained in this study are available and have been submitted to GenBank at the NCBI (PV665019).

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