Research Note: Prevalence and zoonotic concern of *Blastocystis* in farmed chickens in southern China

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ABSTRACT *Blastocystis* is a gastrointestinal protozoan parasite commonly reported in humans and animals globally, including poultry, and it can cause zoonotic transmission of blastocystosis. However, comprehensive information is not available on the prevalence, subtype distribution and zoonotic potential of *Blastocystis* in chickens in China. In this study, a total of 1,000 individual fecal samples of free-range broiler chickens of 4 breeds were collected from 43 farms in 5 cities of Guangdong Province and investigated for the occurrence of *Blastocystis* infection. *Blastocystis* was determined by nested PCR analysis of the small subunit ribosomal RNA (**SSU rRNA**) gene. The overall prevalence was 20.1% (201/1,000) in chicken samples and 69.8% (30/43) in screened farms, and considerable variation in prevalence between farms was evident, with a range of 0 to 76.9%. Population differences of *Blastocystis* in broilers among sites, breeds, and ages were assessed. The highest infection rates were observed in Yangjiang city (35.8%, 38/106), Sanhuang chickens (29.7%, 104/350), and the >80-day-old chicken group (30.5%, 40/131). DNA sequencing and phylogeny analyses identified 2 zoonotic subtypes, ST6 and ST7. A large predominance was observed for ST7, and genetic polymorphisms were confirmed at the intra-ST7 level with the identification of 5 divergent ST7 types. The incidence of both STs varied largely based on the breed, site, farm, and age. This is the first large-scale study to explore the prevalence and genetic characteristics of *Blastocystis* in chickens in China. The widespread distribution and avian adaptation of both zoonotic subtypes were demonstrated. The findings of this study highlight a potential threat to humans and will provide a better understanding of the epidemiology and public health impact of poultry *Blastocystis*.

Key words: *Blastocystis*, prevalence, genetic characteristics, zoonotic, poultry

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INTRODUCTION

Blastocystis is one of the most common protists that infect the gastrointestinal tract of humans and numerous animals, including poultry (Tan, 2008; Maloney et al., 2021). As a gut commensal, *Blastocystis* has a global distribution. Because many infections occur in asymptomatic individuals, the pathogenicity of *Blastocystis* is still under debate (Salehi et al., 2022). The fecal-oral route is the main transmission mode of the parasite, and infection can also result from contaminated water and food (Greige et al., 2018).

Based on sequence polymorphisms of the small subunit ribosomal RNA (**SSU rRNA**) gene, the genus *Blastocystis* in mammals and birds revealed extensive genetic diversity, and 28 of 32 subtypes (**STs**) have been proposed as valid by meeting the current subtype criteria (Higuera et al., 2021). Epidemiological data showed that at least 14 STs (ST1-ST10, ST12, ST14, ST16, and ST24) infecting various animals were observed in humans, implying a crucial zoonotic potential (Greige et al., 2018; Maloney et al., 2021; Salehi et al., 2022). In addition, it has been determined that that zoonotic transmission occurs based on the much higher infection rates of *Blastocystis* reported in animal handlers than in those with no contact with these animals (Wang et al., 2018). Recently, ST6, a subtype generally identified in avians, was confirmed in abattoir staff in Lebanon, thus demonstrating zoonotic infection with *Blastocystis* through repeated or direct contact from chickens to

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handlers (Greige et al., 2018). Moreover, most of the poultry surveyed harbored potentially zoonotic *Blastocystis* STs, and therefore, could serve as reservoirs of human infections (Hublin et al., 2021).

In China, the chicken industry is of major economic importance at present, and the number of chickens raised has been the largest worldwide for many years (https://bg.qianzhan.com/report/detail/a8770a1ded764354.html?v=title). However, literature on the *Blastocystis* status of chickens in China is lacking. Therefore, the aim of this study was to identify the infection state and genetic characteristics of *Blastocystis* in several chicken species or ages in Guangdong Province, southern China, as well as to evaluate the zoonotic transmission potential in this densely populated area.

MATERIALS AND METHODS

Ethics Statement

The protocol of this study was reviewed and approved by the Ethics Committee of Tarim University (approval no. ECTU2018-0026). Before collecting the fecal samples, permission was obtained from all farmers. No specific permits were required for the described field studies. No animals were harmed during the sampling process.

Study Sites and Sample Collection

This study was conducted in five cities of Guangdong Province, namely, Qingyuan, Maoming, Yangjiang, Huizhou, and Shanwei, and samples were supplied by 43 farms that raised free-range broilers. Each farm corresponded to 1 batch and 1 to 3% chickens were surveyed based on random sampling method. A total of 1,000 chickens were sampled from 4 breeds: Sanhuang chicken (n = 350), Ma chicken (n = 362), Grass chicken (n = 211), and Wenchang chicken (n = 77) (Table 1). Three age categories were observed for the animals: <40 d, 40 to 80 d and >80 d (Table 1). Only one fecal sample was used per chicken in this study. Each fecal sample was collected into a 25 ml EP tube using sterilized disposable toothpicks, labeled with basic information (site, farm, age, and breed), placed into a container under cool conditions, and immediately transported to the lab within 48 h for molecular analysis. During sampling, all fecal materials with no contact with the ground were used and collected as quickly as possible after defecation to ensure no environmental contamination. All investigated chickens were apparently healthy in the present study.

DNA Extraction and PCR

Genomic DNA was extracted with the E.Z.N.A. Stool DNA kit (Omega, Norcross, GA) following the manufacturer's instructions. *Blastocystis* was detected by performing nested PCR based on the SSU rRNA gene using RD3 (5'-GGGATCCTGATCCTTCCGCAGGTTCACCTAC-3'), RD5 (5'-GGAAGCTTATCTGGTTGATCCTGCCAGTA-3'), Bla1 (5'-GGAGGTAGTGACAATAAATC-3'), and Bla2 (5'-TGCTTTCGCACTTGTTCATC-3'), which amplified a fragment of approximately 480 bp in length. All PCR amplifications were performed using Tan PCR master mix (Songong, China) and conducted in a GeneAmp System 9700 (Applied Biosystems, Foster, CA). The PCR systems used herein were exactly the same as previously described (Santin et al., 2011). PCR products were examined by 1% agarose gel electrophoresis. Positive PCR products were sequenced at Songong using the ABI PRISM 3730XL DNA Analyzer (Applied Biosystems). Two-directional sequencing was performed to ensure accuracy.

Sequence Analysis and Phylogeny

To determine the *Blastocystis* subtypes, nucleotide sequences obtained in this study were subjected to BLAST searches (http://www.ncbi.nlm.nih.gov/blast/) and then aligned with each other and the reference sequences in GenBank using the program ClustalX 2.1 (http://www.clustal.org/). The phylogenetic relationships of *Blastocystis* subtypes were analyzed by the neighbor-joining (**NJ**) algorithm under the Kimura 2-parameter model in MEGA 7.0 software (http://www.megasoftware.net/). Bootstrap values were evaluated with 1,000 replicates.

Unique representative SSU rRNA sequences gained in this study were deposited in GenBank under accession numbers OL514226 to OL514231.

Statistical Analysis

The infection rates with 95% confidence intervals (**CIs**) were calculated by Wald's method in SPSS version 22.0 (SPSS Inc., Chicago, IL). The $\chi 2$ test was used to analyze the prevalence differences of *Blastocystis* between groups, and a P value of < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Blastocystis infection occurs in numerous animal species worldwide, and the population characteristics of this protist vary based on the host type and geographical location (Hublin et al., 2021). Of the 1,000 chicken samples analyzed in this study, 201 were positive for *Blastocystis*, indicating a total prevalence of 20.1% (Table 1). This finding was slightly lower than that reported for Brazil (33.8%, 43/130) and Lebanon (32%, 71/223) in recent studies (Greige et al., 2018; Maloney et al., 2021) but closer to the actual infection status due to the large sampling number. The highest infection rate was found in Yangjiang city at 35.8% (38/106, $P = 0.004$), and the lowest was found in Huizhou city at 0.9% (2/227, $P = 0$; Table 1). The remaining three cities exhibited similar prevalences ($P > 0.05$): 27% (76/282) in Maoming, 23.5% (69/294) in Qingyuan, and 17.6% (16/91) in

Table 1. Infection and subtype distribution of *Blastocystis* in chickens in southern China.

Factor	Sampling types	No. positive/no. examined (%; 95%Cl)	*P*-value	Subtype (no.)
Regions	Farms			
Qingyuan city	1-1	6/28 (21.4; 6.2−36.6)		ST7-Type2 (4), ST7-Type4 (2)
	1-2	0/17 (0)		-
	1-3	6/17 (35.3; 12.6−58)		ST7-Type1 (6)
	1-4	5/24 (20.8; 4.6−37.1)		ST6 (1), ST7-Type1 (2), ST7-Type2 (2)
	1-5	9/33 (27.3; 12.1−42.5)		ST7-Type2 (7), ST7-Type4 (2)
	1-6	0/22 (0)		-
	1-7	11/22 (50.0; 29.1−70.9)		ST7-Type1 (10), ST7-Type2 (1)
	1-8	0/25 (0)		-
	1-9	12/16 (75.0; 53.8−96.2)		ST7-Type1 (12)
	1-10	1/17 (5.9; 0−17.1)		ST7-Type2 (1)
	1-11	10/13 (76.9;54−99.8)		ST6 (2), ST7-Type1 (1), ST7-Type2 (6), ST7-Type4 (1)
	1-12	0/17 (0)		-
	1-13	9/17 (52.9; 29.2−76.7)		ST7-Type4 (1)
	1-14	0/26 (0)		-
	Subtotal	69/294 (23.5; 18.6−28.3)	0.237	ST6 (3), ST7-Type1 (39), ST7-Type2 (21), ST7-Type4 (6)
Maoming city	2-1	5/25 (20.0; 4.3−35.7)		ST7-Type1 (5)
	2-2	4/25 (16.0; 1.6−30.4)		ST6 (4)
	2-3	2/24 (8.3; 0−19.4)		ST6 (1), ST7-Type1 (1)
	2-4	10/26 (38.5; 19.8−57.2)		ST7-Type1 (8), ST7-Type4 (2)
	2-5	14/28 (50.0; 31.5−68.5)		ST6 (13), ST7-Type4 (1)
	2-6	9/24 (37.5; 18.1−56.9)		ST6 (9)
	2-7	12/29 (41.4; 23.5−59.3)		ST6 (1), ST7-Type1 (8), ST7-Type5 (3)
	2-8	8/49 (16.3; 6−26.7)		ST6 (1), ST7-Type1 (4), ST7-Type2 (1), ST7-Type4 (2)
	2-9	10/26 (38.5; 19.8−57.2)		ST6 (5), ST7-Type1 (3), ST7-Type2 (1), ST7-Type4 (1)
	2-10	2/26 (7.7; 0−17.9)		ST6 (1), ST7-Type1 (1)
	Subtotal	76/282 (27.0; 21.8−32.1)	0.072	ST6 (35), ST7-Type1 (30), ST7-Type2 (2), ST7-Type4 (6), ST7-Type5 (3)
Yangjiang city	3-1	16/28 (57.1; 38.8−75.5)		ST6 (2), ST7-Type1 (7), ST7-Type2 (5), ST7-Type3 (1), ST7-Type4 (1)
	3-2	6/27 (22.2; 6.5−37.9)		ST6 (1), ST7-Type1 (5)
	3-3	6/26 (23.1; 6.9−39.3)		ST6 (2), ST7-Type1 (4)
	3-4	10/25 (40.0; 20.8−59.2)		ST7-Type1 (2), ST7-Type1 (3), ST7-Type3 (1), ST7-Type4 (4)
	Subtotal	38/106 (35.8; 26.7−45)	0.005	ST6 (5), ST7-Type1 (18), ST7-Type2 (8), ST7-Type3 (2), ST7-Type4 (5)
Huizhou city	4-1	1/15 (6.7; 0−19.3)		ST7-Type1 (1)
	4-2	0/16 (0)		-
	4-3	0/31 (0)		-
	4-4	0/16 (0)		-
	4-5	1/15 (6.7; 0−19.3)		ST7-Type1 (1)
	4-6	0/15 (0)		-
	4-7	0/15 (0)		-
	4-8	0/15 (0)		-
	4-9	0/89 (0)		-
	Subtotal	2/227 (0.9; 0−2.1)	0	ST7-Type1 (2)
Shanwei city	5-1	0/15 (0)		-
	5-2	3/17 (17.6; 0−35.8)		ST7-Type4 (3)
	5-3	2/16 (12.5; 0−28.7)		ST7-Type1 (1), ST7-Type4 (1)
	5-4	2/8 (25.0; 0−55)		ST6 (1), ST7-Type1 (1)
	5-5	5/17 (29.4; 7.8−51.1)		ST7-Type1 (5)
	5-6	4/18 (22.2; 3−41.4)		ST7-Type1 (4)
	Subtotal	16/91 (17.6; 9.8−25.4)	reference	ST6 (1), ST7-Type1 (11), ST7-Type4 (4)
Breeds	Days			
Ma chicken	<40	22/215 (10.2; 6.2−14.3)		ST7-Type1 (6), ST7-Type2 (12), ST7-Type4 (4)
	40-80	47/118 (39.8; 31−48.7)		ST6 (1), ST7-Type1 (41), ST7-Type2 (3), ST7-Type4 (2)
	>80	12/29 (41.4; 23.5−59.3)		ST6 (3), ST7-Type1 (2), ST7-Type2 (6), ST7-Type4 (1)
	Subtotal	81/362 (22.4; 18.1−26.7)	0.417	ST6 (4), ST7-Type1 (49), ST7-Type2 (21), ST7-Type4 (7)
Sanhuang chicken	<40	25/60 (41.7; 29.2−54.1)		ST6 (22), ST7-Type1 (1), ST7-Type4 (2)
	40-80	63/262 (24.0; 18.9−29.2)		ST6 (15), ST7-Type1 (33), ST7-Type2 (5), ST7-Type3 (1), ST7-Type4 (9)
	>80	16/28 (57.1; 38.8−75.5)		ST6 (2), ST7-Type1 (7), ST7-Type2 (5), ST7-Type3 (1), ST7-Type4 (1)
	Subtotal	104/350 (29.7; 24.9−34.5)	0.04	ST6 (39), ST7-Type1 (41), ST7-Type2 (10), ST7-Type3 (2), ST7-Type4 (12)
Grass chicken	<40	1/75 (1.3; 0−3.9)		ST7-Type1 (1)
	40-80	1/91 (1.1; 0−3.2)		ST7-Type1 (1)
	>80	0/45 (0)		-
	Subtotal	2/211 (0.9; 0−2.3)	0	ST7-Type1 (2)
Wenchang chicken	<40	0/25 (0)		-
	40-80	2/23 (8.7; 0−20.2)		ST7-Type4 (2)
	>80	12/29 (41.4; 23.5−59.3)		ST6 (1), ST7-Type1 (8), ST7-Type5 (3)
	Subtotal	14/77 (18.2; 9.6−26.8)	reference	ST6 (1), ST7-Type1 (8), ST7-Type4 (2), ST7-Type5 (3)
Total		201/1000 (20.1; 17.6−22.6)		ST6 (44), ST7-Type1 (100), ST7-Type2 (31), ST7-Type3 (2), ST7-Type4 (21), ST7-Type5 (3)

Shanwei (Table 1). In addition, the overall prevalence of *Blastocystis* reached approximately 69.8% (30/43) in the farms screened, which could be compared to the 64.9% (48/74) observed in Lebanon (Greige et al., 2018). Moreover, the carriage rate of this parasite in each infected farm varied greatly, from 5.9% to 76.9% (Table 1). As surveyed in domesticated animals, the variation in *Blastocystis* incidence was related to environmental factors (Hublin et al., 2021; Salehi et al., 2022), suggesting discrepancies in sanitation conditions and feeding management among current geographic regions and farms. However, relevant studies have rarely been conducted in China. Future research should focus on the detection of the drinking water and environment on chicken farms.

In this study, the correlation between *Blastocystis* infection and breed was statistically significant. The Sanhuang chickens were more susceptible to *Blastocystis* than the other breeds, with an infection rate of 29.7% (104/350, $P = 0.04$), and the lowest susceptibility was observed in Grass chickens, with a rate of 0.9% (2/211, $P = 0$; Table 1). For Ma and Wenchang chickens, the infection rates were almost consistent at 22.4 and 18.2% ($P = 0.417$) (Table 1), respectively. However, the reason for the abovementioned differences needs to be further explored. For the age data, infections were highest in chickens aged > 80 d (30.5%, 40/131), followed by those aged 40 to 80 d (22.9%, 113/494), and these rates were much higher than that of chicks aged less than 40 d (12.8%, 48/375, $P < 0.005$). The findings were consistent with results reported in cattle and pigs, which showed that *Blastocystis* infection tended to increase with age in young hosts (Hublin et al., 2021). Thus, the age of an animal may play an extremely important role in *Blastocystis* infection. Additionally, the infection rate of 57.1% (16/28) in Sanhuang chickens aged > 80 d was highest, while no infections were found in Ma chickens of the same age; in contrast, most positive isolates of *Blastocystis* occurred in Ma chickens among the 40- to 80-day-old chickens, with a prevalence of 39.8% (47/118) (Table 1).

BLAST analysis for all isolates in the GenBank databases determined 2 known subtypes, ST6 and ST7, despite the large number of chickens detected in this survey. Each *Blastocystis*-positive sample analyzed corresponded to a single infection by either ST6 or ST7. Of them, ST7 was largely predominant (78.1%, 157/201) in poultry compared with ST6 (21.9%, 44/201) after combining data from farms or breeds (Table 1). Conversely, ST6 was the most common subtype (77.5%, 55/71) in chickens in some countries (Greige et al., 2018; Salehi et al., 2022). The superior divergence of subtypes might result from geographical variations between countries. Sequence analysis showed that genetic polymorphisms were only present in the ST7 isolates in the present study. Of the ST7 isolates, 5 different SSU rRNA sequences with a few nucleotide mutations were identified and clustered with other reported ST7 isolates from multiple hosts by phylogenetic analysis, namely, ST7-type 1 to 5 (Figure 1). Among 201 chicken isolates, ST7-type 1 was the most dominant subtype, with a proportion of 49.8% (100/201). Infections of ST6 (44/201), as the second most common subtype, were mostly consistent with those of ST7-type 2 (31/201) but much higher than those of ST7-type 4 (21/201). In contrast, ST7-type 5 and ST7-type 3 revealed a sporadic incidence in chickens (Table 1). Remarkably, the discrepancy in prevalence among these STs was significant for different categories, including the region, farm, age, and breed (Table 1). In addition, ST7-type 1 was only found in Huizhou city and Grass chickens, which suggested that ST7-type 1 might be the main adaptive subtype of *Blastocystis* in that region or breed. Further verification is required.

To date, subtyping *Blastocystis* has facilitated the identification of 28 recognized STs at the SSU rDNA locus, 11 of which (ST1, ST2, ST4-7, ST9, ST10, ST14, ST25, and ST29) were identified in various bird hosts (Wang et al., 2018; Maloney et al., 2021). Among them, ST6 and ST7 made the major contributions to infect avians, especially poultry, represented the most widely distributed subtypes in avians and were generally considered avian-adapted subtypes (Greige et al., 2018; Wang et al., 2018; Gabrielli et al., 2021; Salehi et al., 2022). Similarly, the results from the current study were similar to those of previous reports in chickens, indicating that chickens were natural hosts for *Blastocystis* ST6 and ST7. However, other subtypes consisting of zoonotic and animal-specific STs (ST10, ST14, ST25, and ST29) were determined occasionally in only Brazilian domesticated chickens (Maloney et al., 2021). The absence of those STs in the present study suggested limited transmission from their susceptible host populations near the chicken farms surveyed.

As potentially zoonotic subtypes, ST6 and ST7 were repeatedly observed and accounted for a small proportion (nearly 9%) of human *Blastocystis* infections in multiple regions, including several provinces of China (Stensvold and Clark, 2016; Li et al., 2021). In a recent study, ST7 was found to be predominate subtype (64%, 16/25) in diarrheal patients in Singapore, further confirming its potential pathogenicity to humans (Deng et al., 2021). Moreover, Guangdong is a huge commercial province with high population density. Thus, it is crucial to investigate the infection status of *Blastocystis* in farmed chickens. The subtype distribution of *Blastocystis* ST6 and ST7 plus high prevalence herein implied a possibility of zoonotic transmission of the protist from chick origin, especially in breeders, butchers, and other individuals having contact with fecal contaminants. Taken together, the 2 STs in chickens may constitute a public health concern based on close contact or environmental contamination in southern China.

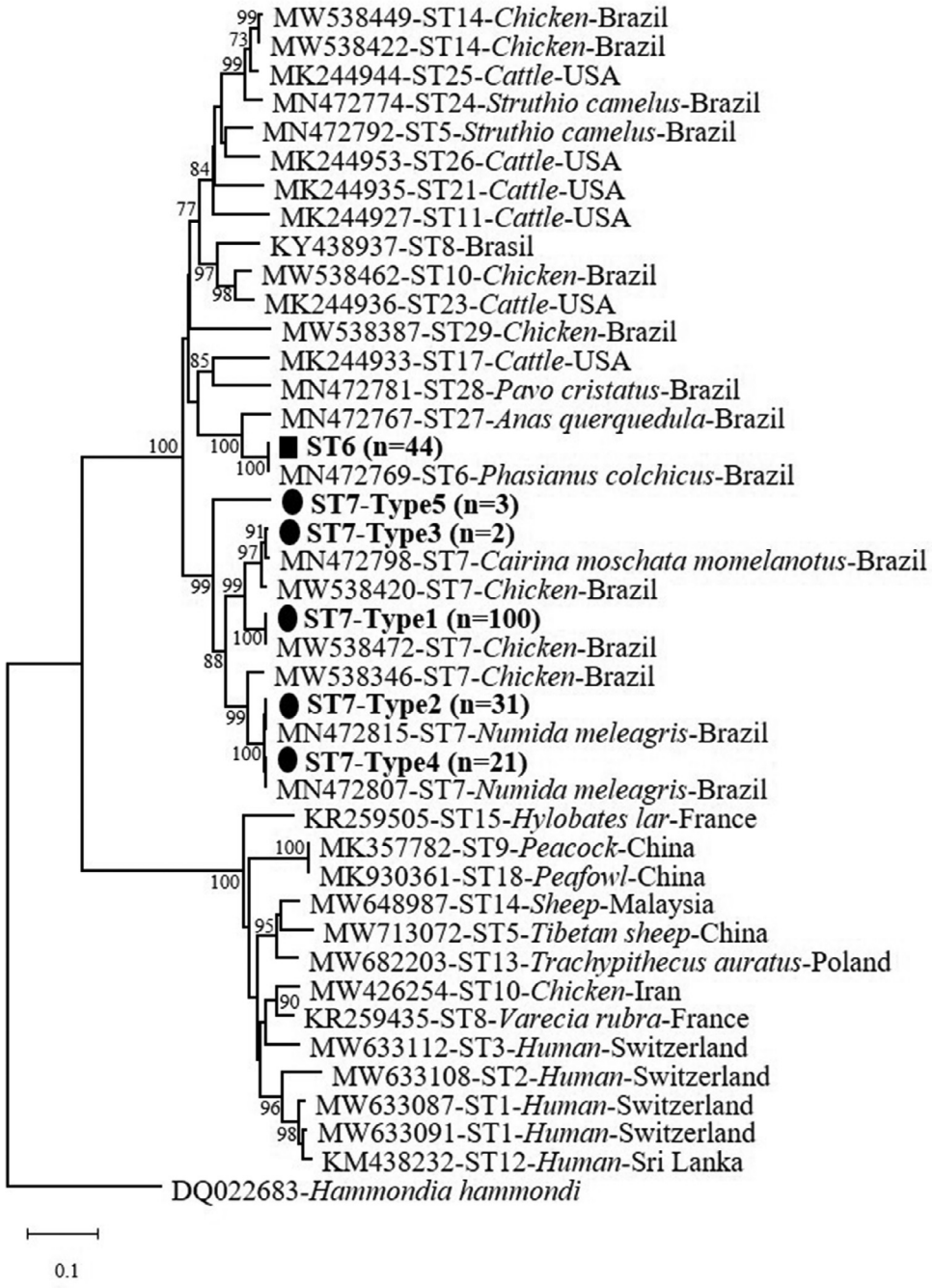

Figure 1. Phylogenetic relationship of the SSU rRNA genes of *Blastocystis* isolates from fecal samples of chickens. Relationships to other known *Blastocystis* subtypes were inferred by the neighbor-joining (NJ) method based on evolutionary distances. Bootstrap values were obtained using 1,000 pseudoreplicates, and those with >50% were shown. *Hammondia hammondi* was used as the outgroup for this tree. Bar = substitutions/site. The round and square icons represent the subtype sequences obtained in this study.

In conclusion, to the best of our knowledge, this is the first large-scale report exploring the prevalence and ST distribution of *Blastocystis* in Chinese chickens using molecular tools. The results showed that 20.1% of the tested chickens were positive of *Blastocystis.* Sequence analysis confirmed the presence of zoonotic ST6 and ST7 infection, and genetic polymorphisms were clarified within ST7 isolates. Chicken region, breed, age, and sampling number influenced the infection rate of both STs. Therefore, the potential of zoonotic transmission from chicken sources cannot be ignored, and further efforts are needed to better understand the infection patterns of *Blastocystis* in the current region.

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DISCLOSURES

The authors declare that they have no competing interests.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.102182.

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