Short Communication

Subtyping of *Blastocystis* infection in common quails (*Coturnix coturnix*) in Xinxiang city of China

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Abstract

Blastocystis is a unicellular anaerobic intestinal protist primarily of various animal species worldwide, and has been reported to inhabit humans as well. *Blastocystis* infections and subtype identification have been documented in China, although infections by the protist in quail remain unknown. Herein, a total of 128 fecal samples were collected from captive quails (*Coturnix coturnix*) in Xinxiang city, central China, and tested for the prevalence and genetic characteristics of *Blastocystis*. Ten (7.8%) samples were positive by PCR amplification and sequencing the barcode regeion of the SSU rRNA gene. Sequence analysis showed that ST7 was the predominant subtype with max identity of 100% to human isolates. Phylogenetic analysis of current isolates corroborated the initial classification, which indicated the potential susceptibility of quail to ST7 and extended its host range in this region. This study is the first to explore *Blastocystis* infection in quail in China, and the findings of human-pathogenic ST7 suggest the possibility of zoonotic transmission to breeders.

Keywords: *Blastocystis*, subtype, genetic characteristics, zoonotic transmission, quail

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Introduction

Blastocystis, one of the most common protists, is commonly reported in the feces of a variety of vertebrate species worldwide, including humans and birds (Deng *et al.*, 2021[a]). Generally, *Blastocystis* inhabits the gastrointestinal tract of hosts and is transmitted via the fecal-oral route. Contaminated food and water are the primary sources of infection (Chai *et al.*, 2020; Deng *et al.*, 2021[b]). Annually, this parasite is believed to colonize the guts of approximately 1 to 2 billion patients globally based on epidemiological surveys (Scanlan and Stensvold, 2013). Recent studies have shown that the occurrence of IBS (irritable bowel syndrome) and IBD (inflammatory bowel disease) are associated with *Blastocystis* infection (Kumarasamy *et al.*, 2018). Infection with *Blastocystis* typically presents as slight intestinal discomfort, including abdominal pain, vomiting, constipation, diarrhea, and flatulence (El-Badry *et al.*, 2018; Stensvold and Clark, 2016). It has been demonstrated that healthy or asymptomatic individuals from different continents can carry *Blastocystis* with a high prevalence, and partial human infections may be due to the zoonotic transmission of *Blastocystis* (Deng *et al.*, 2021[b]; Xiao *et al.*, 2019).

Extensive genetic diversity has been reported within the genus *Blastocystis* (Gentekaki *et al.*, 2017). Based on sequence analysis of the SSU rRNA gene, *Blastocystis* have been classified into 22 different subtypes (STs) (Deng *et al.*, 2021[a]; Stensvold and Clark, 2020). Notably, infection rates associated with different STs are highly variable in either geographical regions or hosts (Clark *et al.*, 2013). To date, at least 10 STs, consisting of ST1-10, ST12 and ST14, are have exhibited zoonotic transmission with varying prevalence (Chai *et al.*, 2020). However, other STs strains are usually exclusively found in a few special animal species, exhibiting some host-specificity (Stensvold and Clark, 2016). Studies conducted in numerous countries show that ST6-7 are the most common STs detected in birds (Deng *et al.*, 2021[b]; Deng *et al.*, 2019; Greige *et al.*, 2018; Salehi *et al.*, 2021[b]; Wang *et al.*, 2018). In addition, ST1-2, ST4, ST5, ST8, ST10, ST14 and ST24 have occasionally been isolated from several bird species (Deng *et al.*, 2019; Maloney *et al.*, 2020; Roberts *et al.*, 2013; Rostami *et al.*, 2020; Stensvold *et al.*, 2009).

In past decades, *Blastocystis* infections have been reported in a variety of avian species, including poultry, pets, and captive wildlife, with different prevalence (Chai *et al.*, 2020; Maloney *et al.*, 2020; Salehi *et al.*, 2021[b]). In China, some *Blastocystis* isolates harboring host-specific adaptations for poultry have been isolated from human patients (Ning *et al.*, 2020). However, no information on the infection and subtype identification of *Blastocystis* in common quails in China is currently available, and its role as reservoirs of human infection remains unclear. This study aimed to estimate the prevalence and genetic characteristics of *Blastocystis* in common quails in central China, and to assess the associated zoonotic risk.

Materials and Methods

Ethical statement: This study was performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Health, China. The protocol of the current study was reviewed and approved by the Research Ethical Committee of the Henan Institute of Science and Technology. Before the initiation of experiments, permission was obtained from farm owners prior to the collection of fecal samples, and no animals were harmed during the sampling.

Sample collection and DNA extraction: A total of 128 samples were collected from a farm that raised common quails in October 2019 in Xinxiang city, central China. Every three quails were housed together in a separate cage, and before cleaning the excrement disc of quail cages, approximately 10 g fresh feces were randomly sampled from the feces one disc per cage as one sample. Each stool sample was collected into a sterile Eppendorf tube and sealed. Furthermore, sterile disposable gloves were used and changed between the collection of each sample to minimize potential sources of contamination among cages. No obvious clinical signs such as diarrhea or apocleisis were observed by farm veterinarians in the sample collection. All fecal samples were transported to the laboratory in a refrigerated sampling box within 24 h of collection, and DNA extraction was performed immediately upon arrival.

Approximately 200 mg feces of each sample were used to extract genomic DNA using the Stool DNA Kit (Omega Bio-Tek Inc., D4015-2, USA) according to the manufacturer's protocol. A total of 200 µl DNA was eluted using elution buffer at 65 °C. The DNA was then stored at -20 °C until the time of PCR analysis.

Subtyping Blastocystis: All extracted DNA was subjected to PCR amplification to screen for the presence of *Blastocystis* infection by targeting a ~600 bp fragment of the barcode region of the SSU rRNA gene, using the primers and cycling parameters reported previously (Scicluna *et al.*, 2006). The PCR reaction was conducted in a reaction volume of 25 µl containing 12.5 µl Taq PCR Master Mix (Sangon, Shanghai, China), 1 µl each primer (0.01 µM), 2 µl genomic DNA, and 8.5 µl double distilled H_2O. A total of 35 amplification cycles were used for the PCR. Reagent grade H_2O was used as a negative control in all PCR runs. Amplicons were resolved using 1% agarose gel electrophoresis, and bands were visualized with ethidium bromide staining. Positive PCR products were bidirectionally sequenced on an ABI 3730 DNA Analyzer (Applied Biosystems, CA, USA).

Phylogenetic analysis and accession numbers: Nucleotide sequences generated here were aligned with each other using the program Clustal X v.2.1 (http://www.clustal.org/), and were then subjected to a BLAST search (http://www.ncbi.nlm.nih.gov/blast/) to identify *Blastocystis* subtypes. Reference sequences were downloaded from the GenBank database (http://www.ncbi.nlm.nih.gov). Before phylogenetic analysis, the obtained sequences and reference sequences were aligned again using the Clustal W algorithm in MEGA 7.0 (http://www.megasoftware.net/) using the default parameters. A neighbor-joining (NJ) tree was constructed to

determine the genetic relationship of current isolates among *Blastocystis* STs in MEGA 7.0. Evolutionary distances were calculated using the Kimura two-parameter model. Bootstrap analysis with 1000 replicates was utilized to assess the reliability of the phylogenetic tree.

The partial nucleotide sequences of the SSU rRNA genes obtained in this study were deposited in GenBank under accession numbers: MW867026-MW867033.

Results and Discussion

Of the 128 samples screened, 10 (7.8%) were *Blastocystis*-positive by PCR, which was confirmed by sequence analysis. The prevalence of *Blastocystis* in this study was similar to that of birds surveyed in Sichuan (8.8%) and Heilongjiang (7.0%) provinces of China (Deng *et al.,* 2021b; Wang *et al.,* 2018). Compared with poultry in the southwest (20.4%) and northwest (15.9%) regions of Iran and Lebanon (32%), the current result was markedly lower (Greige *et al.,* 2018; Rostami *et al.,* 2020; Salehi *et al.,* 2021[b]). Due to studies of quails involving small sample sizes, the highest prevalence of 20% (1/5) was reported in Brazil (Maloney *et al.,* 2020). Likewise, other wild birds infected with *Blastocystis* exhibited a wide prevalence range (0-100%, e.g. 60% in ostriches and 100% in Swan geese) (Roberts *et al.,* 2013; Stensvold *et al.,* 2009).

Adjustment of the alignment windows revealed that the sequences of all 10 isolates were identical to each other, and were identified as ST7 accompanied with max identity of 100% to human isolates from China (FJ809939 and EU082109). Compared to other isolates from different hosts in the GenBank data, anywhere from 1 to 19 nucleotide differences were observed in sequences of quail isolates in the present study. In the phylogenetic tree, all of the *Blastocystis* isolated here were grouped into subtype ST7 of various hosts, and were thus confirmed to be *Blastocystis* ST7 (Fig.1). Previous studies have indicated that ST7 infection occurs sporadically in a few captive wild birds, such as pheasants, swan geese and red crowned cranes (Maloney *et al.,* 2020; Wang *et al.,* 2018), and was frequently detected in domestic chickens (Greige *et al.,* 2018; Rostami *et al.,* 2020; Salehi *et al.,* 2021[b]). Quails can be naturally invaded by ST2, ST4, ST6 and ST7, with a predominance of the latter two subtypes (Iguchi *et al.,* 2007; Maloney *et al.,* 2020; Rivera 2008). However, only ST7 was identified in the present study, suggesting that ST7 was the predominant subtype in quails in that geographic region.

Blastocystis ST7 infecting humans, as a rare subtype, has been reported in recent years in some countries, including China, which showed a zoonotic potential (Kaczmarek *et al.,* 2020; Khaled *et al.,* 2020; Ning *et al.,* 2020; Salehi *et al.,* 2021[a]). Moreover, mammals such as rats, sheep, cattle and guinea pigs also appear to be the hosts of *Blastocystis* ST7, which is suggestive of ST7 transmission between birds and these mammals (AbuOdeh *et al.,* 2019; Iguchi *et al.,* 2007; Salehi *et al.,* 2021[b]). Several studies on the epidemiology of *Blastocystis* have provided evidence suggesting a higher prevalence of *Blastocystis* among animal handlers or owners compared with humans not normally in contact with animals (AbuOdeh *et al.,* 2019; Kaczmarek *et al.,* 2020). Thus, the findings of these studies demonstrate a striking zoonotic risk for handlers in the farm.

In conclusion, this study is the first report on *Blastocystis* infection in quails in China. The identification of ST7 suggests a public health implication for humans. However, the transmission route and host-specificity of *Blastocystis* ST7 in birds in this region requires further investigation.

Consent to Participate: All the authors consented to participate in this study.

Consent to Publish: All the authors consent to publication of this article.

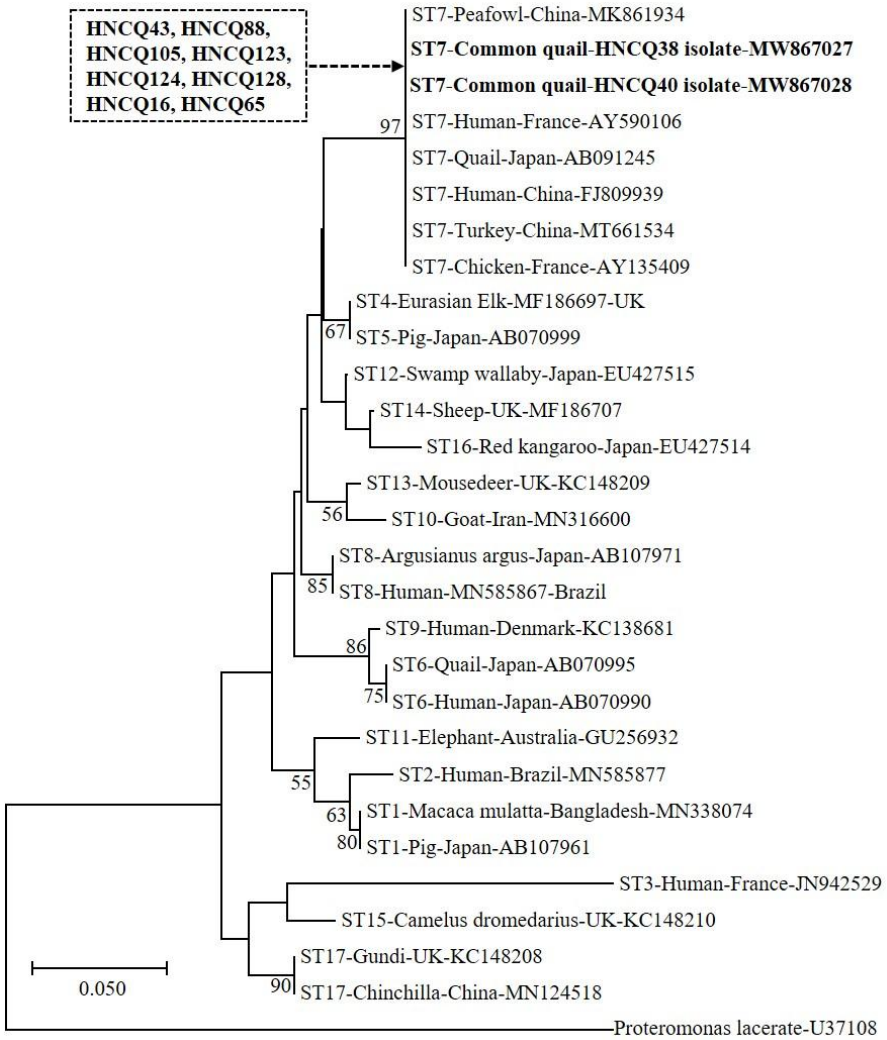

Figure 1 Phylogenetic relationship of the SSU rRNA genes of *Blastocystis* isolates from quail fecal samples. Relationships to other known *Blastocystis* subtypes was inferred by a neighbor-joining (NJ) method based on evolutionary distances calculated using the Kimura two-parameter model. Bootstrap values were obtained using 1,000 pseudoreplicates. Bar=substitutions/site

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Competing interests: The authors declare that they have no competing interests.

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

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