One Health 16 (2023) 100486

Contents lists available at ScienceDirect

One Health

journal homepage: www.elsevier.com/locate/onehlt

First detection of *Blastocystis* sp. in migratory whooper swans (*Cygnus cygnus*) in China

Kaihui Zhang [a,b,c], Ziyang Qin [a,b,c], Huikai Qin [a,b,c], Yinlin Wang [a,b,c], Luyang Wang [a,b,c], Yin Fu [a,b,c], Changjiang Hou [d], Chenxiao Ji [d], Yuan Yuan [d], Longxian Zhang [a,b,c,*]

[a] College of Veterinary Medicine, Henan Agricultural University, Zhengzhou 450046, PR China
[b] International Joint Research Laboratory for Zoonotic Diseases of Henan, Zhengzhou 450002, Henan Province, PR China
[c] Key Laboratory of Quality and Safety Control of Poultry Products (Zhengzhou), Ministry of Agriculture and Rural Affairs, PR China
[d] Sanmenxia Management Office of Henan Yellow River Wetland National Nature Reserve, Sanmenxia 472000, PR China

ARTICLE INFO

Keywords:
Blastocystis
Cygnus cygnus
Zoonotic
Public health

ABSTRACT

Blastocystis is a usual intestinal protist that always found in humans and various animals. Currently, the prevalence of *Blastocystis* in the migratory whooper swan (*Cygnus cygnus*) is unknown. In our research, we aimed to determine the occurrence, prevalence, subtype distribution and dynamic transmission mechanisms of *Blastocystis* in the migratory whooper swan in China. We also assessed the zoonotic potential of *Blastocystis* isolates, as well as possible routes of transmission and impact of this organism on One Health perspective. Fecal samples (n = 770) were collected from whooper swans inhabiting the Sanmenxia Swan Lake National Urban Wetland Park, China. The overall prevalence of *Blastocystis* was 11.6% (89/770). We identified 9 subtypes of *Blastocystis* sp., including 5 zoonotic subtypes [ST1 (Cakir et al., 2019 (8)), ST4 (Selma and Karanis, 2011 (4)), ST5 (Stensvold et al., 2009 (1)), ST6 (Fare et al., 2019 (5)) and ST7(58)] and 3 host-specific subtypes [ST10 (Zhao et al., 2018 (7)), ST14 (Tan et al., 2010 (2)), ST23 (Wang et al., 2018 (3)), and ST25 (Stensvold et al., 2009 (1))]. Subtypes ST4, ST5, ST6, ST10, ST14, ST23, and ST25 were first identified in the whooper swan. Among these subtypes, ST23 and ST25 were identified in birds for the first time, indicating that these subtypes are expanding their host range. So far, this is the first research reporting on the prevalence and subtypes distribution of *Blastocystis* in the migratory whooper swan in China. The findings obtained in this study will provide new insights into the genetic diversity and transmission routes of *Blastocystis*, and the possible public health concerns posed by this organism.

1. Introduction

Blastocystis sp. are anaerobic enteric protozoans commonly detected in the gastrointestinal tracts of humans and a wide range of animals, including asperissodactyls, artiodactyls, rodents, proboscideans, marsupials, non–human primates (NHPs), reptiles, birds, annelids, insects, amphibians, and fish [1–3]. *Blastocystis* cysts are transmitted to humans mainly by the way of ingestion of contaminated water or food, exposure to fecal contamination, and person–to–person contact [4,5]. The clinical manifestations caused by *Blastocystis* are vary from self–limiting abdominal discomfort to chronic persistent diarrhea and skin lesions [6,7]. Clinical symptoms and severity of disease depend on different *Blastocystis* subtypes. A recent study has shown that *Blastocystis* is more usual detected in healthy individuals than in those with diarrhea or other gastrointestinal symptoms; *Blastocystis* is also found in young and immunocompromised individuals [8].

Extensive genetic diversity within the *Blastocystis* have been revealed by PCR–based analyses among epidemiological studies. Among the 22 published *Blastocystis* subtypes (STs 1–17, ST21, and STs 23–26), ST1-ST9 and ST12 have been always detected in humans, with ST1–ST4 more common [9,10]. ST5 has been found in *Perissodactyla* and *Artiodactyla*, ST6 and ST7 have been found in birds, and ST8 has been found in non–human primates (NHPs) [11]. However, ST10 and ST14 are only characterized in specific animal, suggesting host specificity [12]. Several studies have shown that mixed subtypes occur in a variety of animals [11,13,14]. Evolutionary sources of *Blastocystis* genus various are mainly from inter–subtype recombinant and transmission of infection between host species [15,16].

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

https://doi.org/10.1016/j.onehlt.2023.100486
Received 8 September 2022; Received in revised form 7 January 2023; Accepted 7 January 2023
Available online 9 January 2023

Numerous surveys about the prevalence and subtype distribution of *Blastocystis* sp. in various animal have been reported [1–3]. However, whooper swan (*Cygnus cygnus*) has not been investigated, and available data are scarce. Whooper swan is flock–dwelling herbivorous migratory waterfowl, which is migrate timed and directionally, and stop in specific areas [17]. Indeed, migratory whooper swan have long migration paths and can travel through cities, freshwater lakes, low–lying coastal agricultural lands, wet pastures, and forests, stopping to rest in areas that contain bodies of water. Because of the presence of avian influenza (H5N1) and zoonotic intestinal parasites (*Cryptosporidium* spp. and *Enterocytozoon bieneusi*) in migratory whooper swans, so increased concern and worry of humans about the risk of diseases spreading [18–21]. Some studies have shown that whooper swans carry viruses for long–distance transmission, and that the continuous evolution of viruses may be related to the routes of migratory birds [22,23].

Every winter, >10,000 migratory whooper swans stay in Sanmenxia Swan Lake National Urban Wetland Park for spend the winter [24]. However, humans feeding has led to a dramatic increase in the population of whooper swans (Fig. 1). The main migration route of the whooper swan is from Sanmenxia to Yumenkou wetland, then into the Yellow River of Mongolia, and finally to the central and western parts of Mongolia. However, whooper swan at least rest on 40 watersheds and lakes among migration, thus posing a potential threat to public health [25]. In order to protect the health of the whooper swan and avoid potential public health risks, it is necessary to enquire the occurrence of *Blastocystis* in the whooper swan. Therefore, the purpose of present study is to know and confirm the infection status of *Blastocystis* in migratory whooper swan in China. And the possible route of transmission and the significance of *Blastocystis* on public health is also discussed.

2. Materials and methods

2.1. Sample collection

A total of 770 fresh fecal samples were collected from the whooper swans inhabiting the Sanmenxia Swan Lake National Urban Wetland Park in the city of Sanmenxia, located in the middle reaches of Yellow River, China (Fig. 1). Research shows that wintering period includes early, middle and late: October–November (arrival) is the early wintering period, December–January of the following year is the middle wintering period (this period shows the largest number of birds and most stable community structure), and February–March is the late wintering period (during which the birds leave after the completion of their pre–breeding molt) [25]. So that understand the dynamic transmission mechanism of *Blastocystis* in the migratory whooper swan, we collected fresh fecal samples four times according to the wintering period (Table 1). For each sample, in order to avoid environmental pollution, only the central portion of the fresh fecal was collected into a sterile glove, keep in ice box, and sent to our laboratory, stored at 4 °C.

2.2. DNA extraction

For each sample, fecal whole genomic DNA was extracted based on the instructions of E.Z.N.A.R Stool DNA Kit (Omega Bio–Tek Inc., Norcross, GA, USA). The quality of the DNA samples was assessed using Nanodrop One, and high–quality extracted samples were then labeled, sub–packaged into 200 μl sterile centrifuge tubes, and stored at −20 °C.

2.3. PCR amplification

Blastocystis sp. in all samples were detected using nested PCR amplification of the partial small subunit (*SSU*) rRNA gene. The external primers and internal primers as previously described, which produced a PCR product size of 479 bp [26,27]. PCR assays included positive and negative controls and all samples were tested in triplicate. The PCR products were mixed with DNA Green reagent (Tiandz, Inc., Beijing, China) for staining (1:4), and then detected by 1.0% agarose gel electrophoresis for the presence of the target fragment in the PCR amplification products. The PCR amplification products and positive control samples with the presence of the target fragment were sent to Sangon Biotech (Zhengzhou, China) first–generation bidirectional sequencing.

Table 1
Prevalence and subtypes of *Blastocystis* sp. in whooper swans (*Cygnus cygnus*) in Sanmenxia, China.

Collection date	Whole winter	Positive/Total samples (%)	Subtype (no.)
2018.11.17	Early-term	13.1% (31/237)	ST1 (7); ST6 (1); ST7 (17); ST10 (3); ST14 (1); ST23 (1); ST25 (1);
2018.12.06	Mid-term	11.2% (18/161)	ST7 (15); ST6 (3)
2019.03.18	Late-term	8.7% (6/69)	ST6 (1); ST4 (2); ST7 (3);
2019.12.09	Mid-term	11.1% (34/305)	ST1 (1); ST4 (2); ST5 (1); ST7 (23); ST10 (4); ST14 (1); ST23 (2);
Total		11.6% (89/770)	ST1 (8); ST4 (4); ST5 (1); ST6 (5); ST7(58); ST10 (7); ST14 (2); ST23 (3); ST25 (1)

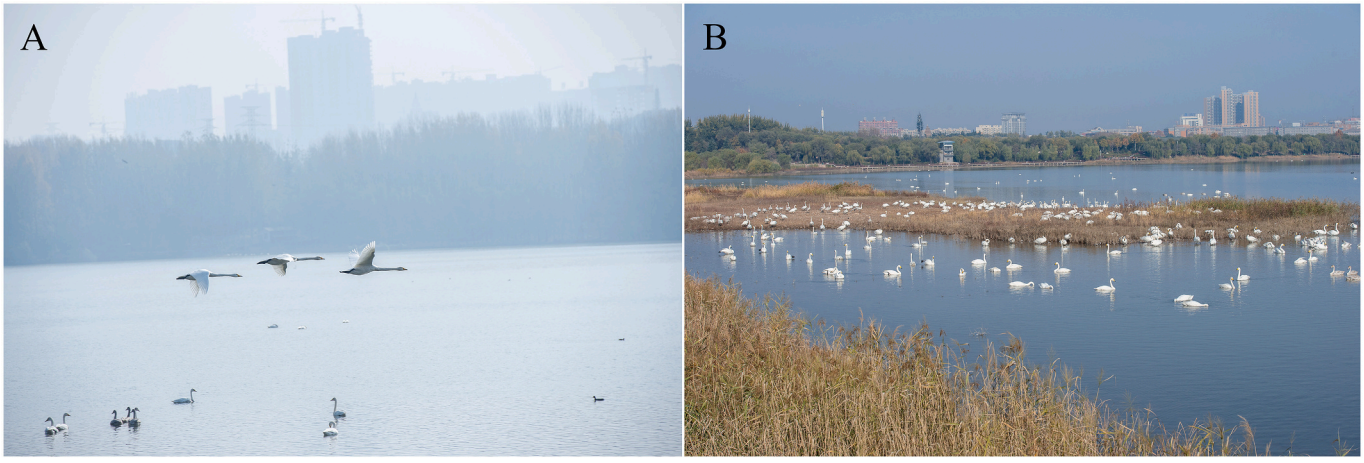

Fig. 1. Migratory whooper swans (*Cygnus cygnus*) at the Swan Wetland Park in Sanmenxia, China.
A: Migratory whooper swan in flight at the Swan Wetland Park in Sanmenxia, China; B: Migrating whooper swans in water and wetlands at the Swan Wetland Park in Sanmenxia, China.

2.4. Sequencing and phylogenetic analysis

After all PCR amplification products and positive control samples with the presence of the target fragment were processed according to the instructions of the Cycle Sequencing Kit (BigDye Terminator v3.1), they were sequenced using the 3730xl DNA Analyzer (ABI PRISMTM). The sequence of the PCR product sample was detected by NCBI Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov) and identified by alignment with a highly homologous reference sequence to identify *Blastocystis* sp. Edited the sequence in Clustal X 2.1 software (http://www.clustal.org/) according to the reference sequence with high homology. The phylogenetic evolutionary tree of *Blastocystis* sp. was constructed and analyzed using MEGA 7.0 software (Kimura two–parameter model, neighbor–joining algorithm) (http://www.megasoftware.net/).

2.5. Statistical analysis

Differences in infection rates with *Blastocystis* sp. between samples collected at different times were analyzed using 95% confidence intervals (CI) and the chi–squared test, which were bast on the SPSS software 22.0 version (SPSS Inc., United States). Significant differences were considered when $P < 0.05$.

3. Results

3.1. Blastocystis prevalence

The overall prevalence in 770 fecal samples collected from whooper swans was 11.6% (89/770). The prevalence differed according to different sampling times. Early–term showed the highest infection rate at 13.1% (31/237), while late–term showed the lowest at 8.7% (6/69). Significant differences ($P > 0.05$) in *Blastocystis* prevalence among the four times of collection, ranging from 8.7% to 13.1% (Table 1).

3.2. Distribution of Blastocystis subtypes

A total of nine *Blastocystis* subtypes were found: ST1 ($n = 8$), ST4 ($n = 4$), ST5 ($n = 1$), ST6 ($n = 5$), ST7 ($n = 58$), ST10 ($n = 7$), ST14 ($n = 2$), ST23 ($n = 3$), and ST25 (n = 1). Among these subtypes, ST1, ST4, ST5, ST6, and ST7 were zoonotic subtypes (Table 1), 7 (ST1, ST6, ST7, ST10, ST14, ST23, and ST25) were found in the 31 *Blastocystis* samples from early–term collection time, 8 (ST1, ST4, ST5, ST6, ST7, ST10, ST14, and ST23) were detected in samples collected during middle–term, and 3 (ST4, ST6, and ST7) were detected in samples collected during late–term. ST25 and ST5 were detected only in the early and mid–collection samples, respectively. ST7 was the predominant subtype, and no mixed subtypes infection was found in present research.

3.3. Phylogenetic analysis of Blastocystis sp.

In our study, we sequenced 89 positive isolates and obtained 13 representative sequences. The sequences obtained are highly homologous to the reference sequence of *Blastocystis* sp. in the GenBank database. (Fig. 2). Eight ST1 isolates produced two variations, and ST1A was the predominant subtype. ST1A was detected in early–term and ST1B was detected in mid–term. Two variations of subtype ST7 were found and subtype ST7A ($n = 47$) was the dominant subtype. ST7A and ST7B were both detected in three periods of winter. Two variations were identified in ST10, and subtype ST10A (n = 4) was the predominant subtype. ST10A was detected in mid–term and ST10B was detected in early–term. Sequence of the ST10A isolate was consistent with that detected in cattle in the USA; the GenBank sequence accession number for this sequence was MK244921. Two ST14 isolates produced two variations: ST14A was detected in early–term and ST14B was detected in mid–term. Phylogenetic analysis revealed more clearly the polymorphisms in the *Blastocystis* isolates detected in present study.

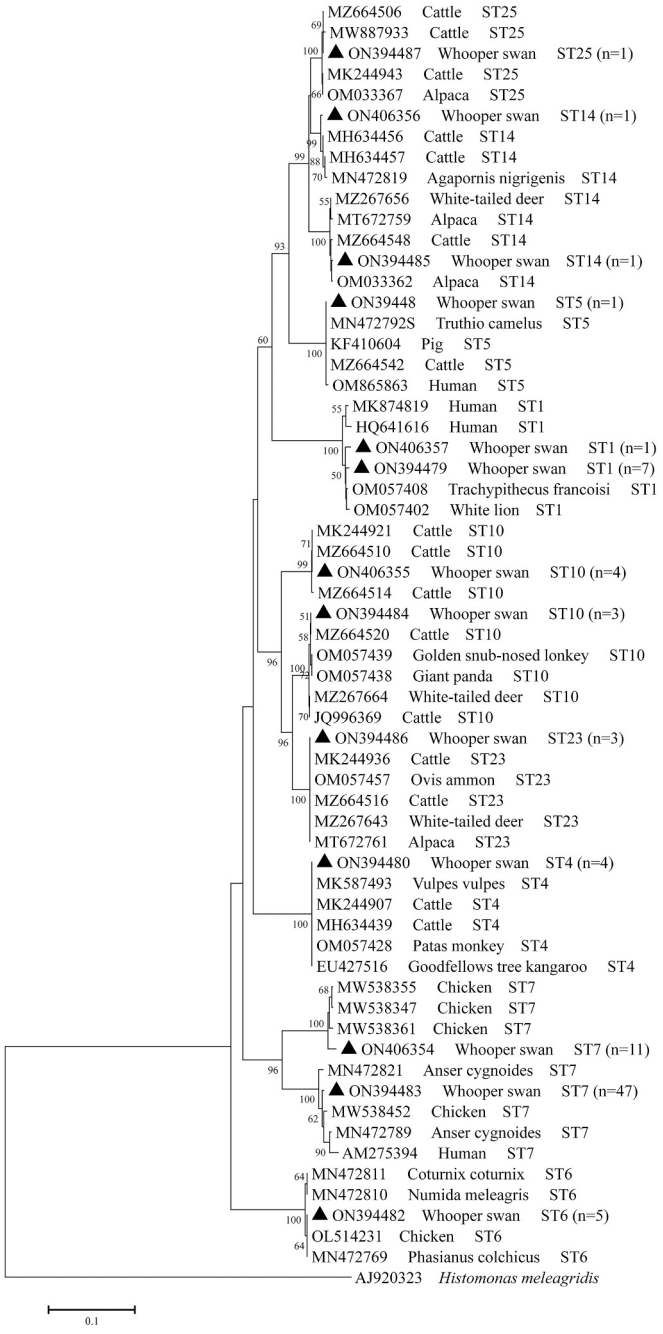

Fig. 2. Neighbor-joining tree of *Blastocystis* subtypes isolated from the whooper swan, based on *SSU* rRNA sequences.
Phylogenetic relationships among the 18S nucleotide sequences of *Blastocystis* subtypes examined in our study, and among other reported *Blastocystis* subtypes. Phylogeny was inferred using the neighbor–joining method. Bootstrap values were obtained using 1000 replicates; those with >50% support are shown on the nodes. The *Blastocystis* sp. identified in this study are designated using filled triangles.

4. Discussion

So far, reports of *Blastocystis* in swan were limited to small population of swan in zoos or parks. Details are as follows: swan goose (*Anser cygnoides*) in Brazil (100%, 1/1), swan in Malaysia (35%, 7/20), swan goose (*Anser cygnoides*) in Spain (16.6%, 3/18), and black swan (*Cygnus atratus*) in China (10.5%, 4/38) [28–31]. It should be noted that all of the previous studies were conducted using small sample sizes. However, several studies have reported on *Blastocystis* in captive and wild birds.

Indeed, *Blastocystis* has been detected in birds (wild and domestic) around the worldwide, with the infection rate ranging from 2.1 to 100% [32]. In our present study, *Blastocystis* prevalence was 11.6% in the whopper swan inhabiting the Sanmenxia Swan Lake National Urban Wetland Park, China. This percentage is within the range reported in other studies conducted in birds, and is lower than the 90% reported in wild bird species in Colombia, 23.4% in bird species (farm, bush land, and zoo) in Australia, and 21% in captive and wild bird species in Brazil [31,33,34]. However, the prevalence observed in present research was higher than those in captive wild birds in China (10.5%) and those in bird species in French zoos (8.6%) [30,35]. These previous studies also show that the infection rate was varies widely, so more studies are needed to decide what factors might influence this variability.

A total of fourteen subtypes (ST1, ST2, ST4, ST5, ST6, ST7, ST8, ST10, ST13, ST14, ST20, ST24, ST27, and ST28) of *Blastocystis* have been detected in birds around the worldwide [32]. In our study, ST1, ST4, ST5, ST6, ST7, ST10, ST14, ST23, and ST25 were detected in the whooper swan. To the best of our knowledge, only ST1, ST3, ST7, and ST8 have been found in swan worldwide [28–31]. Therefore, this is the first research to report on the occurrence of subtypes ST4, ST5, ST6, ST10, ST14, ST23, and ST25 in the swan. ST7 and ST1 were the first and second dominant subtypes found in swan, respectively. The presence of these two subtypes in the swan further confirms the suitability of the swan as host organism for ST7 and ST1. The frequent occurrence of subtypes ST1, ST6, ST7, and ST10 in the swan evaluated in our present study further backs the notion that birds may be common hosts for these subtypes. Isolates ST1A, ST10B, and ST14A, and isolates ST1B, ST10A, and ST14B, were only detected in the early and mid–migration stages, respectively. Hence, migration may promote recombination or reassortment of *Blastocystis* genes, resulting in the generation of numerous new subtypes or isolates. The continuous circulation of *Blastocystis* with seasonal changes in the epidemic areas may also influence this process.

Some studies have shown that ST1 and ST4 are most common in human infection. ST1 and ST4 have also been detected in different animals (NHPs, livestock, birds, wildlife, companion animals, and marine mammals), sewage/wastewater, and surface and drinking water [30–36]. Zoonotic subtype ST5 has been found in ostriches, rodents, pigs and NHPs [4,31,32,35]. ST6 and ST7 are generally regarded to be bird subtypes, which were also occasionally found in human and certain mammals (NHPs, cattle, goats, pigs and dogs) [6–9,32,35,36]. Therefore, ST1, ST4, ST5, ST6, and ST7 pose potential risk for zoonotic transmission or for waterborne transmission. Four non–zoonotic subtypes (ST10, ST14, ST23, and ST25), which are commonly isolated in animals and always regarded animal–specific subtypes, are frequently detected from cattle, deer, yak, alpacas, and goats [3–6,32,37–39]. In our present study, subtypes ST23 and ST25 were found in birds (the whooper swan), indicating that these two subtypes are expanding their host range.

The data obtained thus far suggest that the whooper swan is a natural reservoir of *Blastocystis*, indicating that whooper swans can carry *Blastocystis* for long–distance transmission. The migratory whooper swans have long migration paths and can travel through cities, forests, wet pastures, low–lying coastal agricultural land, and freshwater lakes, stopping to rest in areas that contain bodies of water [17]. These findings indicate that the migration routes of the whooper swan play an important role in the geographic spread of *Blastocystis*. *Blastocystis* cysts can be transmitted to other birds (such as water, ground, and tree birds) and animals (wildlife, livestock, captive farm and companion animals, and marine mammals) via the fecal–oral transmission chain during migration. Additionally, *Blastocystis* is always found in water (such as sewage/wastewater, and surface and drinking water), on vegetables, and in air [36,40,41]. Migratory whooper swans prefer to live in water, and feed on grains and vegetables [17]. Previous studies have suggested that may be more susceptible to budding *Blastocystis* cyst infection due to ground and water birds feeding habits [42]. Roosting sites may serve as reservoirs of *Blastocystis* cysts. *Blastocystis*–infected whooper swans excrete fecal matter when roosting, and *Blastocystis* is then transmitted to susceptible birds via oral ingestion of contaminated water and food (Fig. 3).

Humans can be directly and indirectly infected with *Blastocystis* cysts through contact with the migratory whooper swan. During the migration process, migratory whooper swans can stopover in cities (parks, lakes, and rivers) to feed, rest, and even settle in for the winter [17]. Humans feeding has led to a dramatic increase in the population of whooper swans. However, this behavior can lead to direct contact between humans and the migratory whooper swan, thereby increasing the chance of human contact with migratory whooper swan droppings. Migratory whooper swans also stop in areas containing bodies of water in order to rest and defecate in the water. This activity pollutes the water and can lead to *Blastocystis* transmission to humans through drinking water. When migratory whooper swans forage in low–lying agricultural land and vegetable fields, they can directly contaminate crops and vegetables. Polluted waste and surface water may contaminate food via the process of crop and vegetable irrigation, while fish obtained from polluted water can cause foodborne transmission of *Blastocystis* [43]. Waterborne and foodborne transmission of *Blastocystis* is a public health concern that should not be ignored (Fig. 3).

To date, multiple zoonotic potential of organisms has been reported in migrating swans. For example, avian influenza (H5N1), *Cryptosporidium* spp. *E. bieneusi*, *Blastocystis* sp. (in present study), *Campylobacter* spp., *Streptococcus bovis*, *St. gallolyticus*, *St. pneumoniae*, *Enterococcus faecalis*, *En. faecium*, *Clostridium difficile*, *Clostridium botulinum*, *Clostridium tetani* and *Clostridium perfringens* [18–21]. As the causes of several diseases in humans and animals, the above organisms can persist in the environment for a long time, so they are always considered as indicators of water pollutant monitoring [44–46]. The above results indicate that migratory swans are a potential public health threat. Therefore, there is a need to strengthen the environmental health management of the breeding and wintering grounds of migratory swans to avoid their contamination of water sources and human infections.

5. Conclusions

The migratory whooper swan is a natural reservoir of *Blastocystis*. The nine *Blastocystis* subtypes detected in the migratory whooper swan in China include subtypes ST1 and ST4, which cause >95% of human blastocystosis. These data suggest that migratory whooper swans infected with *Blastocystis* show significant zoonotic potential. The migration process of the migratory whooper swan plays an important role in the geographic spread of *Blastocystis* by promoting the generation of new subtypes or isolates. Additionally, migratory whooper swans may constitute a direct or act as potential mediators, of *Blastocystis* transmission.

Funding

This work was partially supported by the National Key Research and Development Program of China (grant number 2019YFC1605700), the Leading Talents of Thousand Talents Program of Central China (grant number 19CZ0122), the National Natural Science Foundation of China (grant number 32102689), and the Outstanding Talents of Henan Agricultural University (grant number 30501055).

Ethical standards

This study was conducted according to the Regulations for the Implementation of the People's Republic of China on the Protection of Terrestrial Wildlife and Law of the People's Republic of China on the Protection of Wildlife. The research protocol used in this study was reviewed and approved by the Research Ethics Committee of Henan Agricultural University. During our study, no migratory whooper swans were harmed.

Fig. 3. Schematic diagram shows the ecological and public health significance of *Blastocystis* sp. in the migratory whooper swan and major routes of *Blastocystis* transmission.
Possible direct, indirect, waterborne, and foodborne transmission of *Blastocystis* sp. by the migratory whooper swan at the human–animal–environmental interface.

Author statement

Longxian Zhang contributed to the conceptualization and funding acquisition of the study. Kaihui Zhang, Ziyang Qin, and Huikai Qin contributed to the investigation, methodology data curation, formal analysis and writing-original draft writing of the study. Yinlin Wang, Luyang Wang, and Yin Fu contributed to the project administration, resources and software of the study. Changjiang Hou, Chenxiao Ji, and Yuan Yuan contributed to the supervision of the study. All of the authors have read and approved the final version of the manuscript.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

Data availability

All the data generated and analyzed in this study are included in the published manuscript. Nucleotide sequences for the SSU rRNA obtained in this study have been deposited into GenBank (under GenBank accession numbers ON394479–ON394487 and ON406354–ON406357).

Acknowledgments

We thank Let Pub (http://www.letpub.com) for its linguistic assistance during the writing of this manuscript.

References

[1] C.R. Stensvold, H.C. Lewis, A.M. Hammerum, L.J. Porsbo, S.S. Nielsen, K.E. P. Olsen, et al., *Blastocystis*: unravelling potential risk factors and clinical significance of a common but neglected parasite, Epidemiol. Infect. 137 (11) (2009) 1655–1663, https://doi.org/10.1017/S0950268809002672.
[2] K.S.W. Tan, H. Mirza, J.D.W. Teo, B.H. Wu, P.A. Macary, Current views on the clinical relevance of *Blastocystis* spp, Curr. Infect. Dis. Rep. 12 (1) (2010) 28–35, https://doi.org/10.1007/s11908-009-0073-8.
[3] J.G. Wang, B.Y. Gong, F.K. Yang, W.Z. Zhang, Y.H. Zheng, A.Q. Liu, Subtype distribution and genetic characterizations of *Blastocystis* in pigs, cattle, sheep and goats in northeastern China's Heilongjiang Province, Infect. Genet. Evol. 57 (2018) 171–176, https://doi.org/10.1016/j.meegid.2017.11.026.
[4] B. Selma, P. Karanis, Waterborne transmission of protozoan parasites: review of worldwide outbreaks - an update 2004-2010, Water Res. 45 (20) (2011) 6603–6614, https://doi.org/10.1016/j.watres.2011.10.013.
[5] B. Fare, E. Babaei, A. Badirzadeh, T.R. Riabi, A. Abdoli, *Blastocystis*, urticaria, and skin disorders: review of the current evidences, Eur. J. Clin. Microbiol. Infect. Dis. 39 (6) (2019) 1027–1042, https://doi.org/10.1007/s10096-019-03793-8.
[6] H. Yoshikawa, Z.L. Wu, K. Pandey, B.D. Pandey, J.B. Sherchand, T. Yanagi, et al., Molecular characterization of *Blastocystis* isolates from children and rhesus monkeys in Kathmandu, Nepal, Vet. Parasitol. 160 (2009) 295–300, https://doi.org/10.1016/j.vetpar.2008.11.029.
[7] G.H. Zhao, X.F. Hu, T.L. Liu, R.S. Hu, Z.Q. Yu, W.B. Yang, et al., Correction to: molecular characterization of *Blastocystis* sp. in captive wild animals in Qinling Mountains, Parasitol. Res. 117 (1) (2018) 343–344, https://doi.org/10.1007/s00436-017-5692-7.
[8] F. Cakir, M. Cicek, I.H. Yildirim, Determination the subtypes of *Blastocystis* sp. and evaluate the effect of these subtypes on pathogenicity, Acta Parasitol. 64 (1) (2019) 7–12, https://doi.org/10.2478/s11686-018-00002-y.
[9] I. Wawrzyniak, P. Poirier, E. Viscogliosi, M. Dionigia, C. Texier, F. Delbac, et al., *Blastocystis*, an unrecognized parasite: an overview of pathogenesis and diagnosis, Ther Adv Infect Dis. 1 (5) (2018) 167–178, https://doi.org/10.1177/2049936113504754.
[10] R.Y. Tito, S. Chaffron, C. Caenepeel, M.G. Lima, J. Wang, S.S. Vieira, et al., *Blastocystis* population-level analysis of subtype prevalence and variation in the human gut microbiota, Gut. 68 (7) (2019) 1180–1189, https://doi.org/10.1136/gutjnl-2018-316106.
[11] C.R. Stensvold, C.G. Clark, Current status of *Blastocystis*: a personal view, Parasitol. Int. 65 (2016) 763–771, https://doi.org/10.1016/j.parint.2016.05.015.
[12] C. Noel, F. Dufernez, D. Gerbod, V.P. Edgcomb, D.V. Pilar, L.C. Ho, et al., Molecular phylogenies of *Blastocystis* isolates from different hosts: implications for genetic diversity, identification of species, and zoonosis, J. Clin. Microbiol. 43 (1) (2005) 348–355.
[13] R. Abuodeh, S. Ezzedine, M. Madkourm, C.R. Stensvold, A. Samie, G. Nasrallah, et al., Molecular subtyping of *Blastocystis* from diverse animals in the United Arab Emirates, Protist. 170 (5) (2019) 125679–125688, https://doi.org/10.1016/j.protis.2019.125679.
[14] L. Deng, Y.J. Chai, Z.Y. Zhou, H.F. Liu, Z.J. Zhong, Y.C. Hu, et al., Epidemiology of *Blastocystis* sp. infection in China: a systematic review, Parasite. 26 (2019) 41–50, https://doi.org/10.1051/parasite/2019042.
[15] W.B. Karesh, A. Dobson, J.O. Lloyd, J. Lubroth, M.A. Dixon, M. Bennett, et al., Ecology of zoonoses: natural and unnatural histories, Lancet. 380 (9857) (2012) 1936–1945, https://doi.org/10.1016/S0140-6736(12)61678-X.
[16] Z.J. Zhang, J.B. Ma, X.T. Huang, X. Wen, W. Jiang, L. Chen, et al., Population genetic analysis suggests genetic recombination is responsible for increased zoonotic potential of *Enterocytozoon bieneusi* from ruminants in China, One Health. 11 (2020), 100184, https://doi.org/10.1016/j.onehlt.2020.100184.
[17] L. Yuan, X.M. Li, H.X. Yu, L.B. Guo, T.Z. Zhang, Q.J. Gao, et al., The status and conservation of whooper swans (*Cygnus cygnus*) in China, J Forestry Res. 4 (1997) 235–239, https://doi.org/10.1007/BF02875012.
[18] J.H. Liu, H. Xiao, F. Lei, Highly pathogenic H5N1 influenza virus infection in migratory birds, Science. 309 (2005) 1206.
[19] S.H. Li, W.Y. Meng, D.P. Liu, Q.Q. Yang, L.X. Chen, Q. Dai, et al., Migratory whooper swans *Cygnus cygnus* transmit H5N1 virus between China and Mongolia: combination evidence from satellite tracking and Phylogenetics analysis, Sci. Rep. 8 (1) (2018) 7049–7058, https://doi.org/10.1038/s41598-018-25291-1.
[20] K. Wang, A. Gazizova, Y.X. Wang, K.H. Zhang, Y.F. Zhang, Y.K. Chang, et al., First detection of *Cryptosporidium* spp. in Migratory Whooper Swans (*Cygnus cygnus*) in China, Microorganisms 8 (1) (2019) 6–14, https://doi.org/10.3390/microorganisms8010006.
[21] Y.X. Wang, K.H. Zhang, Y.F. Zhang, K. Wang, A. Gazizova, L.Y. Wang, et al., First detection of *Enterocytozoon bieneusi* in whooper swans (*Cygnus cygnus*) in China, Parasit. Vectors 13 (1) (2020) 1–7, https://doi.org/10.1186/s13071-020-3884-y.
[22] Y.L. Si, A.K. Skidmore, T.J. Wang, W.F. Boer, P. Debba, A.G. Toxopeus, et al., Spatio-temporal dynamics of global H5N1 outbreaks match bird migration patterns, Geospat. Health 4 (1) (2009) 65–78, https://doi.org/10.4081/gh.2009.211.

[23] Y. Sakoda, S. Sugar, D. Batchluun, O.T.O. Erdene, M. Okamatsu, N. Isoda, et al., Characterization of H5N1 highly pathogenic avian influenza virus strains isolated from migratory waterfowl in Mongolia on the way back from the southern Asia to their northern territory, Virology. 406 (1) (2010) 88–94, https://doi.org/10.1016/j.virol.2010.07.007.
[24] G.G. Zhang, L.X. Chen, S.H. Li, R.Y. Gao, W.D. Ru, D.P. Liu, et al., The current status of wintering population of whooper swan (*Cygnus cygnus*) at Sanmenxia reservoir region, China, Chinese J Zool 51 (02) (2012) 190–197.
[25] S. Li, Home range and migration of wintering whooper swan (*Cygnus cygnus*) at Sanmenxia reservoir area and spread of HPAI H5N1, Chin. Acad. For. Sci. 16 (3) (2018) 7.
[26] G.B. Böhm, J. Knobloch, B. Walderich, Five subgroups of *Blastocystis* hominis from symptomatic and asymptomatic patients revealed by restriction site analysis of PCR-amplified 16S-like rDNA, Tropical Med. Int. Health 2 (8) (1997) 771–778, https://doi.org/10.1046/j.1365-3156.1997.d01-383.x.
[27] M. Santín, M.M.T. Gómez, A.G. Solano, R. Fayer, Development of a new PCR protocol to detect and subtype *Blastocystis* spp. from humans and animals, Parasitol. Res. 109 (1) (2011) 205–217, https://doi.org/10.1007/s00436-010-2244-9.
[28] C.G. Pérez, P.A. Hitos, D. Romero, M.M. Sánchez, A. Pontes, A. Osuna, et al., Intestinal parasitism in the animals of the zoological garden "Peña Escrita" (Almuñecar, Spain), Vet. Parasitol. 156 (2018) 302–309, https://doi.org/10.1016/j.vetpar.2008.05.023.
[29] A.S. Muadica, P.C. Köster, A. Dashti, B. Bailo, M. Hernández, L. Reh, et al., Molecular epidemiology of *Blastocystis* sp. in Animals Reared by the Aborigines during Wet and Dry Seasons in Rural Communities, Pahang, Malaysia, Se Asian J Trop Med 48 (2017) 1151–1160.
[30] L. Deng, J.X. Yao, H.F. Liu, Z.Y. Zhou, Y.J. Chai, W.Y. Wang, et al., First report of *Blastocystis* in Giant pandas, Red Pandas, and Various Bird Species in Sichuan Province, Southwestern China, Int J Parasitol Parasites Wildl 9 (2019) 298–304, https://doi.org/10.1016/j.ijppaw.2019.06.007.
[31] B.C. Valença, T.C.B. Bomfim, B.R. Teixeira, R. Gentile, S.F.C. Neto, B.S. N. Magalhães, et al., Molecular epidemiology of *Blastocystis* isolated from animals in the state of Rio de Janeiro, Brazil, PLoS One 14 (1) (2019), e0210740, https://doi.org/10.1371/journal.pone.0210740.
[32] J. Hublin, J.G. Maloney, M. Santin, *Blastocystis* in domesticated and wild mammals and birds, Res. Vet. Sci. 135 (2020) 260–282, https://doi.org/10.1016/j.rvsc.2020.09.031.
[33] T. Roberts, D. Stark, J. Harkness, J. Ellis, Subtype distribution of *Blastocystis* isolates from a variety of animals from New South Wales, Australia, Vet. Parasitol. 196 (2013) 85–94, https://doi.org/10.1016/j.vetpar.2013.01.011.
[34] J.D. Ramírez, L.V. Sánchez, D.C. Bautista, A.F. Corredor, A.C. Flórezc, C. R. Stensvoldcd, *Blastocystis* subtypes detected in humans and animals from Colombia, Infect. Genet. Evol. 22 (2014) 223–228, https://doi.org/10.1016/j.meegid.2013.07.020.
[35] A. Cian, S.D. El, M. Osman, R. Moriniere, N. Gantois, V.S. Benamrouz, et al., Molecular Epidemiology of *Blastocystis* sp. in Various Animal Groups from Two French Zoos and Evaluation of Potential Zoonotic Risk, PLoS One 12 (1) (2017), e0169659, https://doi.org/10.1371/journal.pone.0169659.
[36] A.A. Rauff, T.F.H. Meor, N. Shaari, I.L. Lee, The coexistence of *Blastocystis* sp. in humans, animals and environmental sources from 2010–2021 in Asia, Biology (Basel). 10 (10) (2021) 990–1030, https://doi.org/10.3390/biology10100990.
[37] J.G. Maloney, A. Molokin, M. Santin, Next generation amplicon sequencing improves detection of *Blastocystis* mixed subtype infections, Infect. Genet. Evol. 73 (2019) 119–125, https://doi.org/10.1016/j.meegid.2019.04.013.
[38] N. Abarca, S. Ortega, J.G. Maloney, N.S. George, A. Molokin, G.A. Cardona, et al., Molecular detection and characterization of *Blastocystis* sp. and *Enterocytozoon bieneusi* in cattle in northern Spain, Vet Sci. 8 (9) (2021) 191–203, https://doi.org/10.3390/vetsci8090191.
[39] M. Santin, Wide genetic diversity of *Blastocystis* in white-tailed deer (*Odocoileus virginianus*) from Maryland, USA, Microorganisms. 9 (6) (2021) 1943–1958, https://doi.org/10.3390/microorganisms9061343.
[40] M. Isazadeh, D.I. Mirzaii, M. Shaddel, M.M. Homayouni, The prevalence of parasitic contamination of fresh vegetables in Tehran, Iran, Turk, Turkish J Parasitol. 44 (2020) 143–148.
[41] S.A. Nahhas, G. Aboualchamat, Investigation of parasitic contamination of salad vegetables sold by street vendors in City Markets in Damascus, Syria, Food Waterborne Parasitol 21 (2020) 87–91.
[42] J.G. Maloney, A. Molokin, M.J.R. Cunha, M.C. Cury, M. Santin, *Blastocystis* subtype distribution in domestic and captive wild bird species from Brazil using next generation amplicon sequencing, Parasite Epidemiol Control 9 (2020) e00138–e00146, https://doi.org/10.1016/j.parepi.2020.e00138.
[43] N. Gantois, A. Lamot, Y. Seesao, C. Creusy, L.L. Li, S. Monchy, First report on the prevalence and subtype distribution of *Blastocystis* sp. in Edible Marine Fish and Marine Mammals: A Large Scale-Study Conducted in Atlantic Northeast and on the Coasts of Northern France, Microorganisms 8 (3) (2020) 460–478, https://doi.org/10.3390/microorganisms8030460.
[44] L.T. Cao, L.Y. Wang, Y.F. Zhang, K.H. Zhang, R.L. Dong, Y.X. Wang, et al., The gut microbiota diversity of swans from different habitats, Chines J Wildl. 41 (04) (2020) 989–999, https://doi.org/10.19711/j.cnki.issn2310-1490.2020.04.020.
[45] P. Herrera, Y.M. Kwon, S.C. Ricke, Ecology and pathogenicity of gastrointestinal *Streptococcus bovis*, Anaerobe. 15 (1–2) (2009) 44–54, https://doi.org/10.1016/j.anaerobe.2008.11.003.
[46] T. Carmen, A.C. Andrea, R.R. Laura, L.S. Ricardo, D.C. Rosa, T.M. Coque, Antimicrobial resistance in *Enterococcus* spp. of animal origin, Microbiol Spectr 6 (4) (2018), https://doi.org/10.1128/microbiolspec.ARBA-0032-2018.