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Prevalence and Subtype Distribution of *Blastocystis* in Tibetan Sheep in Qinghai Province, Northwestern China

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***Blastocystis* is one of the most common intestinal protists in humans and a great number of animals, including sheep and goats. High prevalence and multiple subtypes of *Blastocystis* have been reported in sheep in several regions of China and elsewhere. However, there is a dearth of knowledge about *Blastocystis* in Tibetan sheep. A total of 761 fecal samples were collected from Tibetan sheep in seven counties of Qinghai Province, northwestern China, and were examined for the prevalence and subtypes of *Blastocystis* using molecular technology based on the partial small subunit ribosomal RNA gene of *Blastocystis*. The overall prevalence of *Blastocystis* in the investigated Tibetan sheep was 7.5% (57/761) using PCR and DNA Sanger sequencing, and differences in prevalence were observed among the ruminants from the seven counties ($P < 0.01$), and across four seasons ($P < 0.01$). Sequence analysis revealed five subtypes (ST14 (57.9%), ST10 (26.3%), ST12 (5.3%), ST21 (5.3%), and ST30 (5.3%)) of *Blastocystis* sp. in these Tibetan sheep, with ST14 as the predominant subtype. To our knowledge, this is the first report of *Blastocystis* colonization in Tibetan sheep.**

Key words: *Blastocystis*; prevalence; subtype; Tibetan sheep; *SSU* rRNA.

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Introduction

Blastocystis, a ubiquitous protist distributed worldwide, colonizes the gastrointestinal tract of humans and a great number of animals (Stensvold et al. 2009; Yoshikawa et al. 2016). The prevalence of *Blastocystis* in vertebrates and fomites varies from 0.5 to 100% (Hirata et al. 2007; Pegelow et al. 1997). Usually, this protist is transmitted through the oral-fecal route, for example, by ingesting contaminated water and food (Taamasri et al. 2000; Yoshikawa et al. 2004). After colonization, the clinical symptoms in animals are often nonspecific; colonization is associated with occasional diarrhea (Goe et al. 2016; Sreekumar et al. 2014; Wang et al. 2014).

Currently, molecular-based analysis is the main method used to identify *Blastocystis*. Based on the genetic variation of the small subunit ribosomal RNA (*SSU* rRNA) locus, at least 27 subtypes have been previously identified in humans and numerous animals (Maloney and Santin 2021; Maloney et al. 2021). Among them, subtypes 1 to 10, subtype 12, and subtype 14 have been found in humans, while all other subtypes have been found only in animals (Khaled et al. 2020; Ramírez et al. 2016; Stensvold and Clark 2016). *Blastocystis* has previously demonstrated a zoonotic potential; thus, animal keepers in close contact with animals are at high risk of *Blastocystis* colonization (Parkar et al. 2010).

Publications in recent years have shown that *Blastocystis* colonization is repeatedly reported in goats, sheep, pigs, cattle, and deer in China (Li et al. 2018; Song et al. 2017a, 2017b; Wang et al. 2018a, 2018b). In sheep and goats, the positive rates of *Blastocystis* range from 0.3 to 94.7% (Table 1), and the dominant subtype is subtype 10 (Deng et al. 2019). To our knowledge, *Blastocystis* colonization has never been reported in Tibetan sheep.

Tibetan sheep, one of the three original sheep species in China, are mainly found in the Qinghai-Tibetan Plateau at an altitude of over 3,000 m (An et al. 2005). For the local herdsman, Tibetan sheep play an important role in economic development, providing daily necessities like meat, milk, wool, skin, and fuel (Zhu et al. 2017). However, due to the harsh environment, most of the farming areas are underdeveloped, and management awareness of the breeding process is weak, which leads to the threat of various parasitic diseases in Tibetan sheep. Recent studies have reported a number of parasitic colonizations in Tibetan sheep, including *Giardia duodenalis*, *Cryptosporidium* spp., *Enterocytozoon bieneusi*, *Toxoplasma gondii*, *Theileria* spp., and *Neospora caninum* (Hao et al. 2020; Nie et al. 2018; Wu et al. 2018; Yin et al. 2015). In this study, we investigated the prevalence and genetic characteristics of *Blastocystis* in Tibetan sheep in Qinghai Province, northwestern China, and our findings are expected to be useful in understanding the colonization of *Blastocystis* in Tibetan sheep.

Results

Prevalence of *Blastocystis* in Tibetan Sheep

Among the 761 Tibetan sheep fecal specimens examined in this study, 57 (7.5%) were positive for *Blastocystis*, as shown by the PCR amplification and sequencing of part of the gene locus *SSU* rRNA (Table 2). Among the seven counties, *Blastocystis*-positive samples were found in only four counties, with the highest (48.1%) and lowest 1.9%) positive rates detected in Haixi and Haibei, respectively; the differences among counties were statistically significant ($v^2 = 244.336$, $df = 6$, $P < 0.01$).

By season, positive samples were detected in summer and autumn. Summer had the highest positive rate (50/212, 23.6%), which was different from that in spring (0/183, 0.0%; $v^2 = 49.416$, $P < 0.01$), autumn (7/262, 2.7%; $v^2 = 48.444$, $P < 0.01$), and winter (0/104, 0.0%; $v^2 = 29.139$, $P < 0.01$).

Subtype Distribution of *Blastocystis* in Tibetan Sheep

The *SSU* rRNA gene of all positive DNA samples was amplified and sequenced. These 57 positive sequences were highly similar (above 95%) to existing subtypes of *Blastocystis* when compared to the GenBank database.

All the sequences of *Blastocystis* samples were aligned with reference sequences using the software Clustal X 1.83. These 1100 bp fragments of the *SSU* rRNA gene were used to construct a phylogenetic tree. After initial phylogenetic tree analysis (Supplementary Material Fig. S1), 33, 15, 3, 3, and 3 sequences clustered in the ST14, ST10, ST12, ST21 and ST30 clades, respectively. Representative sequences of every subtype were chosen to construct the phylogenetic tree of Figure 2.

The divergences between sequence TJ25 and ST14 and ST24 were 2.1% and 3.7%, while the divergence between TJ49 and ST14 and ST24 were 4.7% and 5.2%. After phylogenetic analysis, TJ25

Table 1. Chinese and global prevalence and subtype of *Blastocystis* detected in sheep and goats by molecular methods.

Location	Host	No. of samples	No. of positive samples	Positivity rate (%)	Subtypes	References
Heilongjiang	Sheep	109	6	5.5	ST1, ST5, ST10, ST14	Wang et al. 2018b
Shaanxi	Goats	789	458	58.0	ST10, ST14, ST5, ST4, ST1, ST3, Novel	Song et al. 2017b
Anhui	Sheep	697	22	3.2	ST10, ST14, Novel 1, Novel 2, Novel 3	Li et al. 2018
Anhui	Goats	574	2	0.3	ST1	Li et al. 2018
Jiangsu	Sheep	75	18	24.0	ST5, ST10, ST14	Li et al. 2018
Shandong	Sheep	60	10	16.7	ST10, ST14, Novel 4	Li et al. 2018
Qinghai	Tibetan sheep	761	57	7.5	ST10, ST12, ST14, ST21, ST30	Present study
United Arab Emirates	Sheep	11	7	63.6	ST14, ST10, ST10 + 14	AbuOdeh et al. 2019
UK	Sheep	1	1	100	ST3	Stensvold et al. 2009
UK	Sheep	1	1	100	ST14	Betts et al. 2018
Brazil	Sheep	1	3	33.3	UN	Moura et al. 2018
Libya	Goats	4	38	10.5	ST3, ST7, ST10, Mixed	Alfellani et al. 2013b
UK	Sheep	51	12	40.0	ST10, ST15, Mixed	Alfellani et al. 2013b
Malaysia	Goats	236	73	30.9	ST1, ST6 + 7, ST3 + 6 + 7, ST1 + 6 + 7	Tan et al. 2013
Thailand	Goats	38	36	94.7	ST10, ST12, ST14, UN	Udonsom et al. 2018

Note: UN: unknown.

and TJ49 were located on a separate branch near ST14 but it is possible that they represent a new subype. More sequence data are needed to evaluate this possibility. Similarly, BD3 was also located on a separate branch near ST10 in the phylogenetic tree with the divergence between the BD3 and ST10 (2.3%), ST23 (3.3%). It is also possible that BD3 represents a new subtype.

Among subtypes, ST14 (57.9%, 33/57) was detected with the highest frequency, followed by ST10 (26.3%, 15/57) (Table 2). No mixed subtypes were detected. The genetic diversity analysis showed that the nucleotide diversity of ST10, ST12, ST14, and ST30 was 0.02411, 0.01120, 0.02526, and 0.00321, and the haplotype diversity was 0.971, 1.000, 0.953, and 1.000 respectively. Among them, two sequences of ST21 were identical. The genetic diversity of ST14 exhibited the highest genetic diversity indices followed by ST10 and ST12, while the haplotype diversity was not different among the three subtypes.

Discussion

Blastocystis is a common protist widely distributed globally, colonizing a wide range of species. Based on published surveys of positive rates, the number of peoples colonized by *Blastocystis* is expected to be more than one billion worldwide (Andersen and Stensvold 2016). As an important parasitic protozoan, *Blastocystis* was reported as early as the 1900s (Alexeieff 1911; Brumpt 1912; Lynch 1917). However, its pathogenicity was suggested relatively late by Zierdt and Tan in 1976, who reported it as causing gastrointestinal symptoms such as abdominal pain and diarrhea (Zierdt and Tan 1976). After that, *Blastocystis* received widespread attention. In

Table 2. Occurrence and subtype of *Blastocystis* in Tibetan sheep in Qinghai Province.

Variable	No. of samples	No. of positive samples (%)	Subtype (n)				
			ST10	ST12	ST14	ST21	ST30
Location							
Xining	164						
Haibei	212	4 (1.9)	3		1		
Golog	51						
Hainan	124						
Huangnan	107	7 (6.5)	3	1	3		
Haixi	51	21 (41.2)	5	2	10	1	3
Yushu	52	25 (48.1)	4		19	2	
Season							
Spring	183						
Summer	212	50 (23.6)	12	2	30	3	3
Autumn	262	7 (2.7)	3	1	3		
Winter	104						
Total	**761**	**57 (7.5)**	**15**	**3**	**33**	**3**	**3**

Spring: Mar. 1st to May 31st; Summer: Jun. 1st to Aug. 31st; Autumn: Sep.1st to Nov. 30th; Winter: Dec. 1st to Feb. 30th.

the last decades, many more reports of *Blastocystis* have been published globally, increasing our understanding of this protist.

In the present study, molecular analysis of fecal specimens showed a 7.5% positive rate for *Blastocystis* in Tibetan sheep in Qinghai Province, northwestern China. Relevant studies of *Blastocystis* in Caprinae have been previously conducted only in sheep and goats. This relatively low positive rate which we detected by molecular methods was similar to that observed in sheep from Heilongjiang (5.5%) and Anhui (3.2 %), and in goats from Anhui (0.3%) and Libya (10.5%), but is lower than in sheep from Jiangsu (24.0%), Shandong (16.7 %), the United Arab Emirates (63.6%), UK (40.0%), and Brazil (33.3%), and in goats from Malaysia (30.9%), Shaanxi (58.1%), and Thailand (94.7%) (Table 1). Many factors can contribute to the variable positive rates, such as the sample size. For instance, the number of samples examined in Brazil, the United Arab Emirates, and the UK was 3, 11, and 12, respectively. These sample sizes were far lower than that of the present study. Other factors including economic status, geographical factors, and breeding pattern could also influence the positive rate.

The results of the present study suggest that the prevalence of *Blastocystis* in Tibetan sheep is seasonally variable, reaching 23.6% in summer, compared to 2.7% in autumn. Previous studies also observed the seasonal prevalence of *Blastocystis* in cattle, yak, and wild boar in Korea and China (Lee et al. 2018, 2020; Ren et al. 2019). The highest positive rates were detected in autumn in wild boars, which was different to the other animals. The seasonal pattern of *Blastocystis* colonization in Tibetan sheep is consistent with that in humans, with the positive rate substantially higher in summer than in other seasons (Amin 2006; El Safadi et al. 2016; Haider et al. 2012; Suresh and Smith 2004). The study of Ithoia et al. (2011) indicates that higher temperature and humidity are conducive to *Blastocystis* transmission in summer.

In addition to the seasonal pattern, another important variable is geography. In this study, the positive rate of *Blastocystis* was also different across the regions of Qinghai Province. Given that the Tibetan sheep are managed under the same grazing conditions, the differences might be due to the herd size and changes in the ecological environment (i.e., the altitude difference among the seven sampling sites was more than 1800 m). Due to the unique environment, the Tibetan sheep usually take free grazing, and the herd (age composition, sex ratio, health status, etc.) changes dynamically. In sample collection, the number of fecal samples also varies in different regions and seasons. Although the data have been corrected by the chi-square test (v2 test), the herd remained a confounding factor that contribute to the significant differences in sea-

sonality and geography due to the above reasons. However, the specific reasons require further research.

Currently, *Blastocystis* is classified into 30 subtypes based on the diverse gene sequences in *SSU* rRNA. To date, sixteen known subtypes have been found in sheep and goats, including ST1-ST7, ST10, ST12, ST14, ST15, ST21, ST23-ST26 (Shams et al. 2022; Tan et al. 2013). Among them, ST10 is the predominant subtype. In the present study, three subtypes (ST10, ST12, and ST14) commonly found in animals were isolated from Tibetan sheep, with ST14 as the predominant subtype, unlike previous studies. ST14 has been detected in many animals, including livestock (cattle, sheep, goat, and yaks), and herbivores (camels, giraffe, alpacas, bushbuck, mouflons, and common eland) (Alfellani et al. 2013a; Cian et al. 2017; Wang et al. 2018a; Zhao et al. 2017). Three positive samples were identified as ST12, which has been reported in animals like giraffe and gray kangaroos in Western Australian zoos (Parkar et al. 2010), cattle and goats from Thailand (Udonsom et al. 2018), yaks from Qinghai province (Ren et al. 2019), takin, giraffe, Lechwe water buck, and Mongolian Wild Ass in the Qinling Wildlife Park (Zhao et al. 2017). The other subtypes (ST21 and ST30) have been mainly detected in ruminants, including *Odocoileus virginianus* (white-tailed deer) (Maloney et al. 2021), cattle and *Kobus ellipsiprymnus* (water buck), which are close to Tibetan sheep phylogenetically. However, ST12 has also been detected in three human fecal samples in Bolivia, from asymptomatic patients in close contact with sheep and llamas (Ramírez et al. 2016). More recently, Khaled reported that ST10 and ST14 were detected for the first time in healthy school children's fecal samples in Senegal (Khaled et al. 2020). In Qinghai, there has been no report of *Blastocystis* molecular data in the human population until now. Only one report detected ST12 in humans in Yunnan province in China (Teng et al. 2018). ST10, ST12 and ST14 were also discovered in yaks in Qinghai (Ren et al. 2019). Tibetan sheep are yet to be identified as potential sources of transmission (animal to animal).

Conclusions

To our knowledge, the present study is the first to demonstrate the prevalence and subtype distribution of *Blastocystis* in Tibetan sheep in Qinghai Province, northwestern China. The overall positive rate of *Blastocystis* was 7.5%, with significant differences across regions and seasons. Five distinct subtypes (ST10, ST12, ST14, ST21 and ST30) were identified, with ST14 as the predominant subtype, and ST12, ST21, ST30 were detected for the first time in sheep.

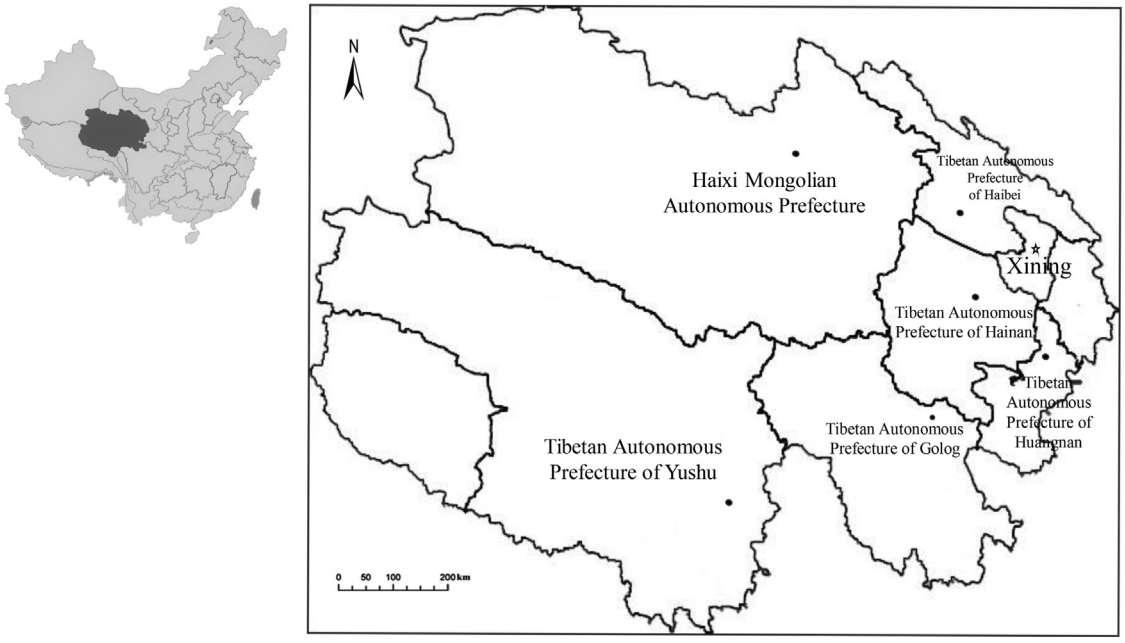

Figure 1. Geographical distribution of fecal sampling counties of Tibetan sheep in Qinghai Province, northwestern China.

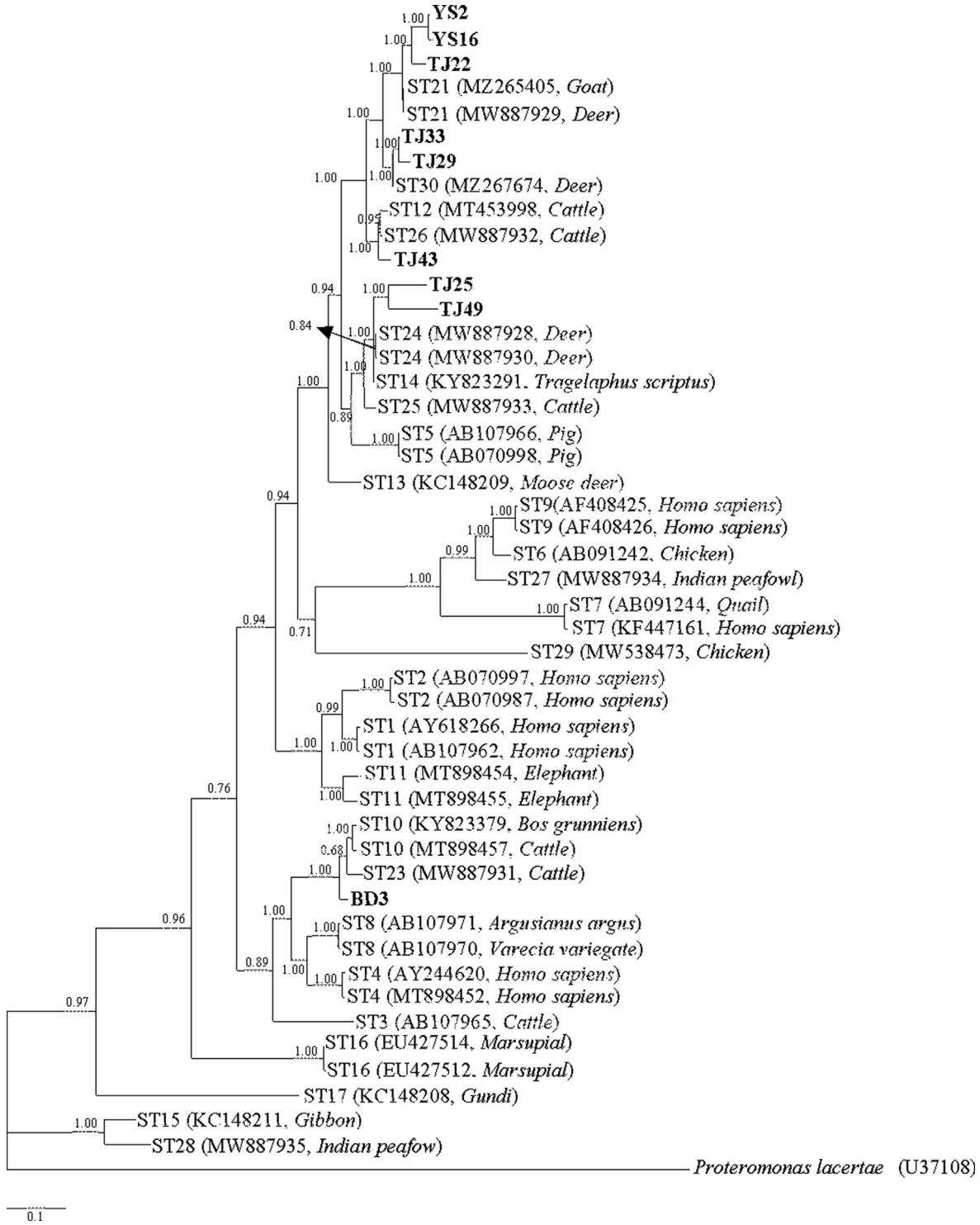

Figure 2. Molecular phylogenetic analysis of nucleotide sequences on *Blastocystis SSU* rRNA genes using Bayesian analysis. Accession numbers used for the sequences are listed inside parentheses. Representative new sequences are marked with bold letters. The phylogenetic tree was rooted to *Proteromonas lacertae* (U37108).

Methods

Specimen collection: A total of 761 fresh fecal specimens were randomly collected from Tibetan sheep over four seasons and in seven different counties of Qinghai Province, northwestern China (Fig. 1). All of the Tibetan sheep were grazing without adverse clinical symptoms and the age difference was relatively small. Because the Tibetan sheep were raised in natural pasture, we collected the top layers of the fecal material immediately after defecation, thus avoiding the part in contact with the ground. Tibetan sheep was numbered before sampling and only one fecal sample was collected per animal. Each sample was placed in a clean plastic bag labeled with collection site and date. All samples were transferred to the laboratory under cool conditions, placed in a standardized laboratory Falcon tube (15 ml) with 2.5% potassium dichromate, and then stored at 4 °C until molecular analysis (within one month).

DNA extraction and PCR: Approximately 0.5 g of a fecal sample was placed in a 2 ml centrifuge tube for multiple rounds of centrifugation at 13,000 × *g* for 1 min with distilled water until the 2.5% potassium dichromate was washed out. DNA was then extracted from the washed fecal material using the Stool DNA Kit (OMEGA, China), according to the manufacturer's instructions. Finally, the purified DNA was eluted using 100 ll elution buffer. Extracted DNA samples were stored at −20 °C prior to analysis via nested PCR for *Blastocystis*.

A ∼ 1,100 bp region of the *SSU* rRNA gene was amplified from the DNA extracted from the stool samples using nested PCR. The reaction was performed in a 25 ll mixture containing: 15.875 ll ddH_2O, 1 ll of genomic DNA, 1 ll of each primer (10 lM), 2.5 ll 10 × TaKaRa *Taq* Buffer (Mg^{2+} free) (20 mM), 2.0 ll dNTPs Mixture (2.5 mM), 1.5 ll $MgCl_2$ (25 mM), and TaKaRa *Taq* 0.125 ll (5U/ll) (TaKaRa Bio Inc., Tokyo, Japan). The primers, RD3: GGGATCCTGATCCTTCCGCAGGTTCACCTAC, and RD5: GGAAGCTTATCTGGTTGATCCTGCCAGTA were described by Clark (1997). PCR conditions were 94 °C for 5 min; 30 cycles at 93 °C for 1 min, 65 °C for 1.5 min, and 72 °C for 2 min; and 16 °C for 2 min. The second amplification used the primer F1: GGAGGTAGTGACAATAAATC, and R2: ACTAGGAATTCCTCGTTCATG (Wong et al. 2008). And the online primers sensitivity test, Prime BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) showed that all known subtypes can be amplified, except ST15, ST16, ST17, and ST28. For example, the binding position of primers (F1, R2) in the ST24 (MW887930) were 447∼466 and 1538∼1558, and in the ST25 (MW887933) were 458∼477 and 1550∼1570, respectively. For the second amplification, the first product was used as a template. The reaction conditions were the same as the first amplification except that the annealing temperature was 49 °C. All PCR reactions were performed using the Gradient PCR instrument (AG22331, Germany, Eppendorf). PCR products were detected with 1.0% agarose gel electrophoresis, stained with ethidium bromide, and visualized under a transilluminator. All positive secondary PCR samples were sent directly to Sangon Biotech Co., Ltd., Shanghai (China) for Sanger sequencing.

Sequence analysis and phylogeny: PCR amplicons of an expected size were sequenced in both directions, ensuring the accuracy of the sequencing results. Raw sequences were then integrated using DNAStar 5.0 (Burland 2000), and the assembled sequences were submitted to Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/) and amended by eye with BioEdit (Hall 1999). Then, the corrected sequences and reference sequence downloaded from GenBank were manually aligned with Clustul X 1.83 (Thompson et al. 1997). Next, the subtypes of *Blastocystis* in the Tibetan sheep were determined via Bayesian analysis using MrBayes 3.1.1 software (Huelsenbeck and Ronquist 2001). Bayesian analysis used four Markov chain Monte Carlo (MCMC) strands, 1,000,000 generations, with trees sampled every 100 generations. In addition, we analyzed the intra-subtype genetic diversity within ST10, ST12, and ST14 using the software DnaSP ver. 5.10.01 (Librado and Rozas 2009). The *SSU* rRNA gene sequences obtained in this study were deposited in GenBank under accession numbers MW269694-MW269726, MW269728-MW269736, and MW269740-MW269754.

Statistical analysis: Overall differences in *Blastocystis* positive rates among seasons and counties were compared using the v^2 test with SPSS Statistics V21.0 (IBM Corp. New York, NY) for Windows. Differences were considered significant at $P < 0.05$.

CRediT authorship contribution statement

Fan Yang: Investigation, Data curation, Writing – original draft, Writing – review & editing. **Jing-min Gou:** Resources, Data curation. **Bing-ke Yang:** Resources. **Jia-yue Du:** Investigation. **Hui-zhong Yao:** Investigation. **Mei Ren:** Data curation. **Qing Lin:** Conceptualization, Funding acquisition.

Data availability

Data will be made available on request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary Material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.protis.2023.125948.

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