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Cryptosporidium spp., *Enterocytozoon bieneusi,* and *Giardia duodenalis* from animal sources in the Qinghai-Tibetan Plateau Area (QTPA) in China

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ABSTRACT

Cryptosporidium spp., *Enterocytozoon bieneusi*, and *Giardia duodenalis* are globally ubiquitous infectious parasitoses in humans and animals. The purpose of the present study was to determine the distribution and genetic diversity of *Cryptosporidium* spp., *Enterocytozoon bieneusi*, and *Giardia duodenalis* from animal sources in different regions of QTPA in China. A total of 405 fresh fecal specimens from seven domestic animals were collected in Qinghai, Yunnan, and Tibet. The overall prevalence of *Cryptosporidium* spp., *E. bieneusi* and *G. duodenalis* was 3.0% (n = 12), 19.8% (n = 80) and 5.7% (n = 23), respectively. Four *Cryptosporidium* species (*C. andersoni*, *C. xiaoi*, *C. bovis*, and *C. ryanae*) were identified and *C. bovis* was firstly identified from camel. Ten genotypes of *E. bieneusi* were identified in the present study, including eight known genotype (BEB6, CAM2, CHG2, CAM1, COS I, J, CHS8, and CHG3), and two novel genotypes (YAK1 and PN). Genotype CAM2 was detected in horse, yak, and mongolian sheep for the first time and the novel genotype YAK1 was grouped into the human-pathogenic group 1. *G. duodenalis* assemblage E was common in all animal species, and the human-pathogenic assemblage A was only detected in yaks and camels. Our results elucidate the occurrence and genetic diversity of three zoonotic pathogens from different animals and regions in QTPA, which could act as potential zoonotic reservoirs. More areas and larger number of samples are required to assess the potential risk of cross-species transmission in this region.

1. Background

Cryptosporidium spp., *Enterocytozoon bieneusi*, and *Giardia duodenalis* have been reported to be the most common intestinal pathogens that infect both humans and animals worldwide [1]. The main transmission route of these pathogens was fecal-oral route, mainly through consumption of contaminated food and water [2–4]. Infected humans might show typical symptoms of gastroenteritis, especially in immunocompromised individuals, which have severe symptoms and a higher risk of infection [5–7]. So, it is necessary to uncover genetic characteristics of *Cryptosporidium* spp., *E. bieneusi* and *G. duodenalis* and elucidate their epidemiological features in order to implement effective therapeutic treatments.

At present, species and genotypes of *Cryptosporidium* spp. have been identified by analysing the small subunit (SSU) rRNA gene. And about 30 species and more than 60 genotypes have been reported [8,9]. Globally, *E. bieneusi* consists of nine groups (Group 1–9), including zoonotic group 1 and several host-specific groups [1,10]. For *G. duodenalis*, eight assemblages (A to H) have been characterized up to now. Among them, assemblage A and B have been detected in both humans and animals, whereas the remaining assemblages seem to be specific for non-human host [1,11].

As known, Qinghai-Tibetan Plateau Area (QTPA) is the largest plateau with high average altitude, low average annual temperature, and changeable climate [12]. There are a variety of domestic livestock in the QTPA including yak, Tibetan sheep, cattle, mongolian sheep, goat, camel, and horse. However, only a few investigations of the presence of *Cryptosporidium* spp. and *G. duodenalis* have been reported in several animal sources Qinghai [8,13,14]. More studies are required to compare and identify the species/subtype, genotype, assemblage of

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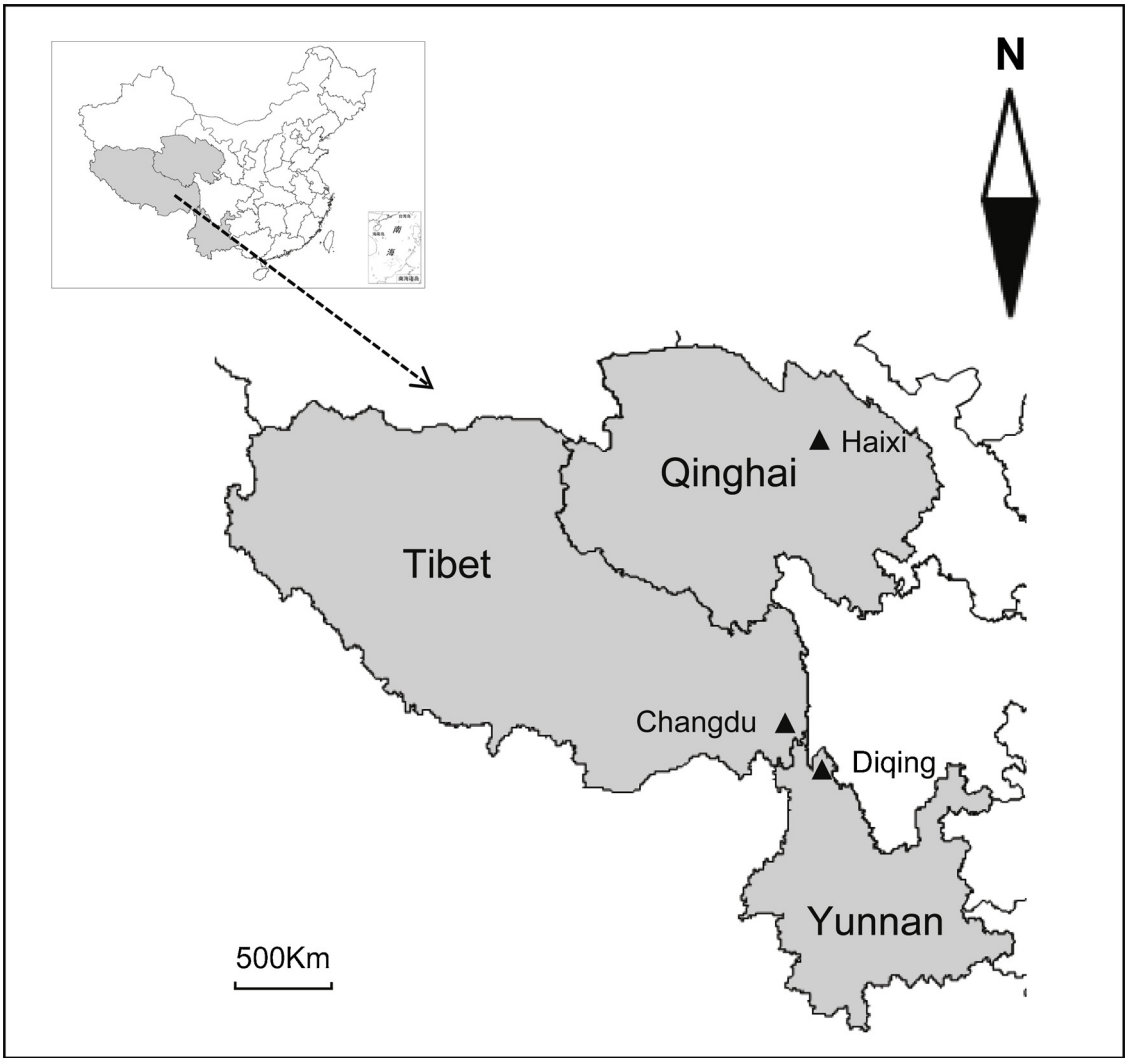

Fig. 1. Investigated locations at which fecal specimens were collected in this study. ▲investigated locations.

Cryptosporidium spp., *E. bieneusi* and *G. duodenalis* in a variety of domestic animal sources at different altitudes (Qinghai, Tibet, and Yunnan) of QTPA.

2. Material and methods

2.1. Sampling sites and specimen collecting

During May 2018 to July 2018, a total of 405 fresh fecal specimens were collected from Haixi in Qinghai Province (36°79′N, 99°09′E, average altitude 3000 m), Changdu in Tibet Province (29°49′N, 98°62′E, average altitude 4000 m) and Diqing in Yunnan Province (28°05′N, 99°77′E, average altitude 3300 m) in the Qinghai-Tibetan Plateau Area (QTPA) in China (Fig. 1). These samples were collected from several animal sources, including Horse (n = 32), Camel (n = 40), Yak (n = 101), Cattle (n = 57), Tibetan sheep (n = 78), Goat (n = 59) and Mongolian sheep (n = 38) without obvious clinical signs. Fresh droppings were collected from each animal using sterile centrifuge tube, placed in a cooler with ice packs and transported to the laboratory for further study. This work was approved by the Animal Ethics Committee of the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences [15].

2.2. DNA extraction and nested PCR amplification

Genomic DNA was extracted from 200 mg fecal samples using a commercially available kit (TIANamp Stool DNA Kit, TIANGEN BIOTECH, BEIJING) following the manufacturer's protocol. In order to obtain high quality and high yield of DNA, a bead-beating step (3000 rpm, 1 min using TGrinder H24 Tissue Homogenizer, TIANGEN BIOTECH) required for lysis of the resistant cysts and oocysts of protists was included and the lysis temperature was increased to 95 °C other than 70 °C. Extracted DNA was stored at −20 °C until PCR analysis. For screening *Cryptosporidium* species/subtype, previously described nested PCR assays were used to amplify the small subunit ribosomal RNA (SSU rRNA) gene [16]. The prevalence and genotypes of *E. bieneusi* were determined using nested PCR amplification of the internal transcribed spacer (ITS) gene [17]. Assemblages of *G. duodenalis* were identified with the beta giardin (*bg*) gene as described by Sulaiman et al. [18]. Vazyme Taq DNA polymerase (Vazyme Biotech Co., Ltd) was used for all PCR amplifications. Positive (DNA extracted from the *C. xiao*, *E. bieneusi* BEB6 genotype and assemblage E of *G. duodenalis*) and negative controls were included in all PCR tests. All secondary PCR products were detected by 1% agarose gel electrophoresis with ethidium bromide stained.

2.3. Sequencing and phylogenetic analysis

All secondary PCR products from positive samples were bi-directionally sequenced at BGI Sequencing (Beijing, China). The obtained sequences were firstly subjected to BLAST searches and then aligned with each other and reference sequences using ClustalX 1.83 software. The representative nucleotide sequences of this study have been deposited in the GenBank database under accession number MK841323-MK841237 for *Cryptosporidium* species/subtype, MK843236-MK843249 for *E. bieneusi* genotypes and MK862309-MK862315 for *G. duodenalis* assemblages. Phylogenetic analysis was constructed by using the neighbor-joining method executed in with Kimura 2-parameter model MEGA6 [19]. The robustness of the tree topology was assessed with 1000 bootstrap replicates.

2.4. Statistical analysis

Differences in infection rates were calculated by the chi-square test using SPSS 25.0 (SPSS). P values < 0.05 were considered statistically significant.

3. Results

3.1. Occurrence of Cryptosporidium spp., E. bieneusi and G. duodenalis

In the present study, the overall prevalence of *Cryptosporidium* spp., *E. bieneusi* and *G. duodenalis* was 3.0% (n = 12, 95%CI: 1.3–4.6%), 19.8% (n = 80, 95%CI: 15.9–23.6%) and 5.7% (n = 23, 95%CI: 3.4–7.9%). *Cryptosporidium* spp. was only found in three types of domestic farm animals, camel (6/40, 15.0%, 95%CI: 3.4–26.6%), Tibetan sheep (4/78, 5.1%, 95%CI: 0.1–10.1%) and yak (2/101, 2.0%, 95%CI: -0.8-4.7%). The prevalence rate of *E. bieneusi* ranged from 9.0% to 45.0% for different animal sources. The highest prevalence was in camel (18/40, 45.0%, 95%CI: 18.8–43.3%), followed by mongolian sheep (16/38, 42.1%, 95%CI: 25.7–58.6%), goat (11/59, 18.6%, 95%CI: 7.0–24.5%), cattle (10/57, 17.5%, 95%CI: 7.4–27.7%), horse (5/32, 15.6%, 95%CI: 2.3–28.9%), yak (13/101, 12.9%, 95%CI: 6.2–19.5%) and Tibetan sheep (7/78, 9.0%, 95%CI: 2.5–15.5%). For *G. duodenalis*, mongolian sheep (6/38, 15.8%, 95%CI: 3.6–27.9%), horse (3/32, 9.4%, 95%CI: -1.3-20.1%), camel (3/40, 7.5%, 95%CI: -1.0–16.0%) and yak (6/101, 5.9%, 95%CI: 1.3–10.6%) had higher infection rates than other species (Table 1). Mixed infection was identified in eleven specimens, as 6 samples (4 camel samples, 1 yak sample and 1 Tibetan sheep sample) were positive for both *Cryptosporidium* spp. and *E. bieneusi*, the remaining five samples (3 mongolian sheep samples and 2 yak samples) were positive for both *E. bieneusi* and *G. duodenalis*. Notably, significant higher infection rates of *Cryptosporidium* spp., *E. bieneusi* and *G. duodenalis* were found in Qinghai (average altitude 3000 m) and Yunnan (average altitude 3300 m) than Tibet (average altitude 4000 m) ($P < 0.01$) (Table 2). However, no correlation between the other two parasitic protozoa infection and region ($P > 0.05$) was found.

3.2. Distribution and genetic characterization of Cryptosporidium species/subtype

All positive PCR products of SSU rRNA region were successfully sequenced. By sequence analysis, four species were identified out of 12 *Cryptosporidium* spp. isolates, including *C. bovis* (n = 2) in camel, *C. ryanae* (n = 1) in yak, and *C. xiaoi* (n = 4) in Tibetan sheep, with *C. andersoni* (n = 5) in both camel and yak. Among them, *C. xiaoi* and *C. andersoni* shared the largest percentage of *Cryptosporidium* spp. isolates (75%, 9/12). Interestingly, this is the first study to report the presence of C. *bovis* DNA in camels. The subsequent molecular phylogeny analysis based on SSU rRNA gene revealed that 5 representative nucleotide sequences were grouped with the subtype *C. andersoni*, *C. ryanae*, *C.*

Table 1
Detection and subtype distribution of *Cryptosporidium* spp., *E. bieneusi* and *G. duodenalis* in different types of domestic farm animals in the Qinghai-Tibetan Plateau Area (QTPA) in China. The novel sequences obtained in the present study are highlighted in bold.

Animal	Location	No. of specimens	*Cryptosporidium* spp.		*E. bieneusi*		*G. duodenalis*	
			No. of positives (%)	Species (no.)	No. of positives (%)	Genotypes (no.)	No. of positives (%)	Assemblages (no.)
Horse	Qinghai,Haixi	32	0	–	5 (15.6)	**CAM2 (5)**	3 (9.4)	E5(3)
Camel	Qinghai,Haixi	40	6 (15.0)	*C.andersoni* (4) ***C.bovis* (2)**	18 (45.0)	CAM1 (8), CAM2 (7), BEB6 (3)	3 (7.5)	**E5 (2), A (1)**
Yak	Qinghai,Haixi, Yunnan,Diqing, Tibet,Changdu	101	2 (2.0)	*C.andersoni* (1), *C.ryanae* (1)	13 (12.9)	BEB6 (11), **CAM2 (1), YAK1 (1)**	6 (5.9)	E1 (2), E (3), A1 (1)
Cattle	Qinghai,Haixi, Yunnan,Diqing	57	0	–	10 (17.5)	BEB6 (1), COS I (3), J (5), **PN (1)**	3 (5.3)	E2 (1), E5 (2)
Tibetan sheep	Qinghai,Haixi, Tibet,Changdu	78	4 (5.1)	*C.xiaoi* (4)	7 (9.0)	CHS8 (3), COS I (4)	1 (1.3)	E8(1)
Goat	Qinghai,Haixi, Yunnan,Diqing	59	0	–	11 (18.6)	CHG2 (9), CHG3 (2)	1 (1.7)	E(1)
Mongolian sheep	Qinghai,Haixi	38	0	–	16 (42.1)	BEB6(13), **CAM2(2)**, CHG3(1)	6 (15.8)	E5 (5), E8 (1)
Total		405	12 (3.0)	*C.andersoni* (5), *C.bovis* (1), *C.ryanae* (1), *C.xiaoi* (4)	80 (19.8)	BEB6 (28), CAM1 (8), CAM2 (15), YAK1 (1), COS I (7),J (5), CHS8 (3), CHG3 (3), CHG2 (9), PN (1)	23 (5.7)	A (1), A1 (1), E (4), E1 (2), E2 (1), E5 (12), E8 (2)

Table 2
The occurrence of *Cryptosporidium* spp., *E. bieneusi* and *G. duodenalis* in different regions of Qinghai-Tibetan Plateau Area (QTPA) in China.

Region	Average altitude (m)	*Cryptosporidium* spp.		*E. bieneusi*		*G. duodenalis*	
		Prevalence (%)	P value	Prevalence (%)	P value	Prevalence (%)	P value
Qinghai	3000	4.6 (95%CI*:1.9-7.3)	0.052	23.8 (95%CI:18.4-29.3)	**0.001**	6.3 (95%CI:3.2-9.4)	0.693
Yunnan	3300	1.1 (95%CI:-1.1-3.4)	0.339	20.7 (95%CI:12.0-29.4)	**0.007**	4.6 (95%CI:0.1-9.1)	0.889
Tibet	4000	0 (95%CI:0-0)	Ref	6.3 (95%CI:0.8-11.8)	Ref	5.1 (95%CI:0.1-10)	Ref

* CI: confidence intervals.

bovis, and *C. xiaoi*.

3.3. Distribution and genetic characterization of E. Bieneusi genotypes

DNA sequence analyses of the ITS gene of *E. bieneusi* revealed the presence of ten genotypes from 80 different animals, including eight known genotypes (genotype BEB6, CAM2, CHG2, CAM1, COS I, J, CHS8, and CHG3), and two novel genotypes (named YAK1 and PN). In the present study, the most prevalent genotypes were BEB6 (35.0%, 28/80), CAM2 (18.8%, 15/80), CHG2 (11.3%, 9/80), CAM1 (10.0%, 8/80), and COS I (8.8%, 7/80). The sequences of the two novel genotypes consisted of mutation and deletions which differed at 2 or 3 nucleotide positions (0.54 and 0.91% difference) from the sequence of known genotypes. And, the two novel genotypes were firstly identified in yak and cattle. By phylogenetic analysis of the ITS gene, 14 isolates of *E. bieneusi* recovered in our study were grouped into three genotypes (Group1 g, Group 2, and Group 8) (Fig. 2). The novel genotype YAK1 fell into the human-pathogenic Group 1. In contrast, the six known genotypes (BEB6, CHG2, COS I, J, CHS8, and CHG3) and one novel genotype (PN) clustered into the cattle host-specific Group 2. And the other two genotypes (CAM1 and CAM2) clustered together into Group 8.

3.4. Distribution and genetic characterization of G. duodenalis assemblages

All 23 *G. duodenalis*-positive specimens were detected and successfully sequenced. Two *G. duodenalis* assemblages (A and E) were revealed for the *bg* gene: 21 were identified as assemblage E (including E, E1, E2, E5, and E8) and 2 as human-pathogenic assemblage A (A and A1). Meanwhile, phylogenetic relationships indicated that 7 representative nucleotide sequences were grouped with the assemblage A and E.

4. Discussion

To explore the role of animal sources in the epidemiology of these pathogens, we characterized the prevalence and species/subtype, genotype, assemblage of *Cryptosporidium* spp., *E. bieneusi* and *G. duodenalis* in a variety of domestic animals at 3 altitudes level of QTPA. Many factors may contribute to the differences between investigations, including biogeographic, age, and sex distribution, health status of animals, number of samples, season of sample collection, and method of detection [20]. Accurate characterization of these three pathogens in animals is critical to assess the potential risk of cross-species transmission and implement effective control strategies.

The past literatures show that infection rates of *Cryptosporidium* spp. vary in humans, domestic animals and wildlife resources between and within countries worldwide [1,5,9,10]. In the investigated areas of QPTA, *Cryptosporidium* spp. was found in three mammal species (yak, Tibetan sheep, and camel) with infection rates ranging from 2.0 to 15.0%, while other ruminants were negative in our study. Similar prevalence was found in other animals in Qinghai [21,22], Guangdong [23], Papua New Guinea [24], and Greece [25]. However, much higher prevalence was identified in several provinces (Qinghai, Inner Mongolia, and Sichuan) of China [22,26,27], Norway [28], Poland [29], Australia [30], and Brazil [31]. *C. andersoni* and *C. parvum* infections have been only reported in several case reports in camel [32–34]. In the present study, high occurrence of *Cryptosporidium* spp. was detected in camel and *C. bovis* DNA was firstly identified from camel. Three species (*C. andersoni*, *C. xiaoi*, and *C. bovis*) identified in our study can be found in human occasionally [35], which might have the potential for zoonotic transmission. In addition, infection rate of *Cryptosporidium* spp. in Qinghai was higher than Yunnan and Tibet (without significant correlation), which may be caused by biogeographic and number of samples.

In this study, *E. bieneusi* was found in seven animal sources with infection rates ranging from 9.0 to 45.0%, and the overall prevalence was 19.8%. Infection rates of *E. bieneusi* in sheep and goats in different provinces of China can vary drastically from 4.4% to 62.5% [20], and the infection rate in this study is lower than the majority of published rates, except for mongolian sheep (42.1%). The prevalence of *E. bieneusi* in cattle (17.5%) was concurred with other reports published in Xinjiang [36] and Shaanxi [37], but higher than Heilongjiang [38], and Henan and Shandong [39]. To our knowledge, there is no other report on *E. bieneusi* infection in camel other than studies conducted by Qi et al. [40] in Bactrian camels in Xinjiang. In their study, 30.0% of Bactrian camel fecal specimens were *E. bieneusi*-positive, which was lower than our study (45.0%). In several studies performed in horses and grazing horses elsewhere, infection rates of *E. bieneusi* vary from 17.3% to 30.9% [41–43], which was higher than our study (15.6%). Ten genotypes of *E. bieneusi* were identified in the present study, including eight known genotype (BEB6, CAM2, CHG2, CAM1, COS I, J, CHS8, and CHG3), and two novel genotypes (YAK1 and PN). CAM1 and CAM2 genotypes were previously only identified in camels and CAM1 was the most predominant genotype in Bactrian camels in Xinjiang [40]. However, genotype CAM2 was detected in horse, yak, and mongolian sheep for the first time, which suggested that there was a higher potential risk of cross-species transmission in this region. The novel genotype YAK1 was grouped into group 1 g which belongs to the human-pathogenic group 1, implying that yaks may be a potential source of human microsporidiosis. In addition, significantly higher infection rate of *E. bieneusi* was found in low altitude (Qinghai and Yunnan) than high altitude (Tibet) ($P < 0.01$), indicated that altitude was an important factor affecting the prevalence of *E. bieneusi* in this region.

The infection rate of *G. duodenalis* in our study (ranging from 1.3 to 15.8% in different animals) is similar to documented reports in Gansu, Inner Mongolia, Heilongjiang, and Qinghai of China [1,44–46], but lower than the majority of published rates in other countries (ranging drastically from 1.5% to 55.6%) [11]. No significant correlation of *G. duodenalis* infections was found in different altitudes. *G. duodenalis* infection in ruminants has a worldwide distribution and assemblage E is most common subtype [11,13]. Meanwhile, two zoonotic assemblages A and B have been also identified in sheep, black-boned sheep and goats, yaks, and horse [21,46–48]. In our study, all animal species were

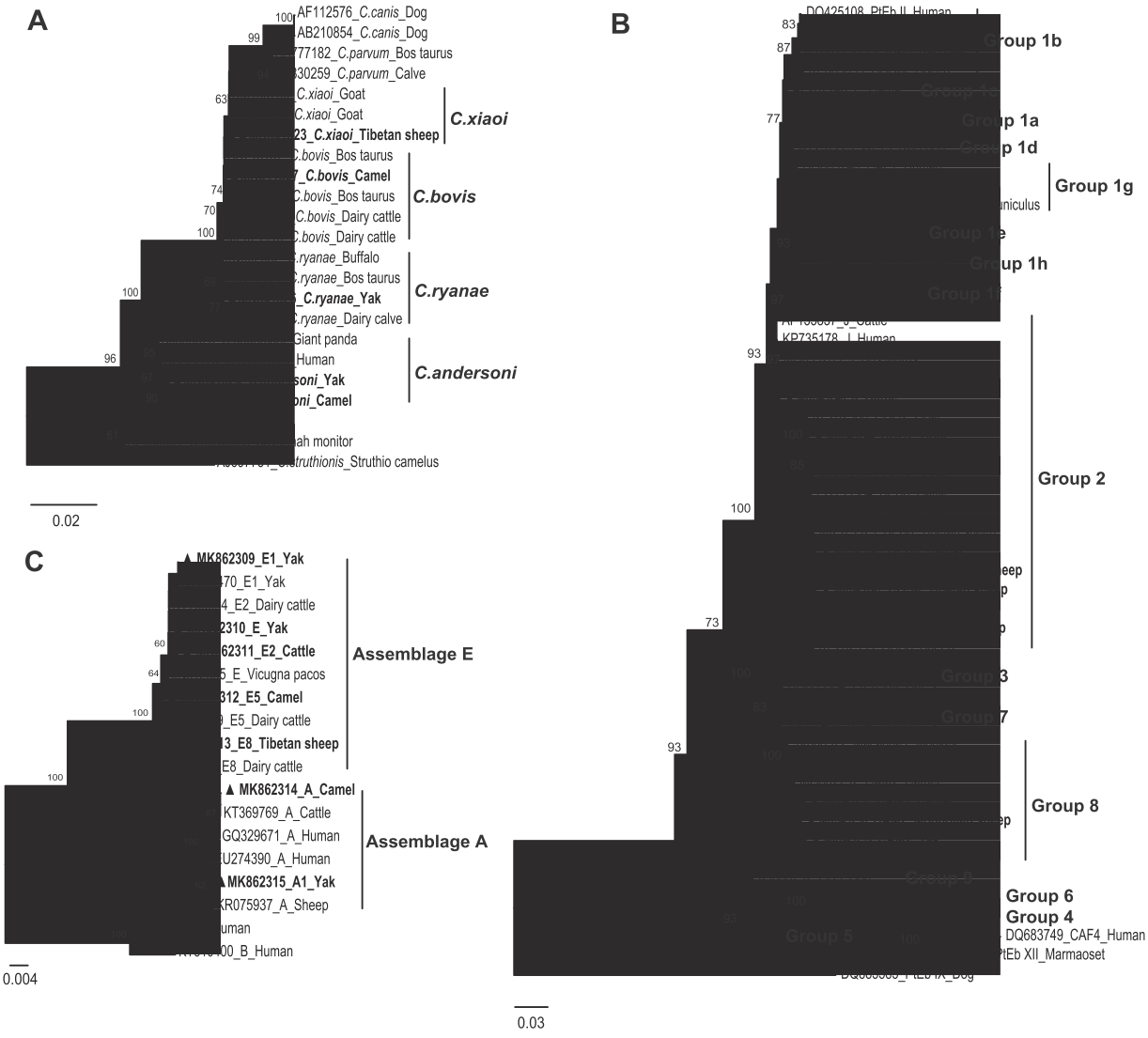

Fig. 2. Phylogenetic relationships of SSU rRNA nucleotide sequences of *Cryptosporidium* spp. (A), ITS nucleotide sequences of *E. bieneusi* (B) and *bg* nucleotide sequences of *G. duodenalis* (C) identified in the present study and reference subtypes. The molecular phylogenetic trees were constructed by using the neighbor-joining method with Kimura 2-parameter model. The genotypes identified in this study are indicated by ▲and highlighted in bold.

positive for *G. duodenalis* assemblage E, and the human-pathogenic assemblage A was only detected in yaks and camels. Our findings constitute the first report of the *G. duodenalis* infection (assemblage E and A) in camels. Considering the presence of human-pathogenic assemblage A of *G. duodenalis* in QPTA, more attention should be paid to assess the public health risk.

5. Conclusions

To our knowledge, our findings firstly provide useful data on the prevalence and genetic diversity of *Cryptosporidium* spp., *E. bieneusi* and *G. duodenalis* in animal sources in different altitudes of QPTA in China. Four *Cryptosporidium* species were identified, with *C. bovis* being observed in camels for the first time. The high frequency and wide genetic diversity of *E. bieneusi* (including eight known genotype and two novel genotypes) was identified and novel genotype YAK1 belongs to the human-pathogenic group 1. Furthermore, *G. duodenalis* assemblages E and A were identified in the present study. Our results suggested that animal sources in QPTA could act as potential zoonotic reservoirs. More areas and larger number of samples are required to better understand the epidemiological features of *Cryptosporidium* spp., *E. bieneusi* and *G. duodenalis* in QPTA in China.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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