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First report on the molecular detection of *Enterocytozoon bieneusi* in livestock and wildlife around Qinghai Lake in the Qinghai-Tibetan Plateau area, China

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ABSTRACT

Enterocytozoon bieneusi is considered to be a microsporidial species of humans and animals in the worldwide. Limited data have been reported on the prevalence and genotypes of *E. bieneusi* in livestock and wild animals around Qinghai Lake in the Qinghai-Tibetan Plateau area, which shares water sources, grasslands, and harsh climate with high altitudes. In this study, fecal samples from 110 Tibetan sheep, 128 yaks, 227 wild birds, 96 blue sheep (*Pseudois nayaur*) and 268 Przewalski's gazelle (*Procapra przewalskii*) around Qinghai Lake were collected, and then tested for *E. bieneusi* by PCR and sequencing analysis based on the ribosomal internal transcribed spacer. Among them, ten (9.09%) samples from Tibetan sheep, five (3.91%) from yaks, five (2.20%) from wild birds, one (1.04%) from wild blue sheep and two (0.75%) from Przewalski's gazelle were positive for *E. bieneusi*. Among sheep, there were nine *E. bieneusi* genotypes, including two known genotypes (BEB6 and J), and seven novel genotypes (named CHS18-CHS24). From yaks, four genotypes were identified, including two known ones (BEB4 and J) and two novel genotypes (named CHN15 and CHN16). While in wild animals, eight genotypes were found, including five different genotypes from wild bids, with three known genotypes (EbpC, J and NCF2), two novel genotypes (named CHWB1 and CHS24), and two genotypes from Przewalski's gazelle, with one known genotype J and one novel genotype CHWPG1, and one novel genotype CHWBS1 from blue sheep. According to the phylogenetic analysis, five isolates belonged to group 1, and the others were clustered into group 2. This study provides unique data on the epidemiological reports and potential risk factors for *E. bieneusi* in both domesticated livestock and wild animals around Qinghai Lake in the Qinghai-Tibetan Plateau area; it is important to better understand the molecular epidemiology and zoonotic potential of *E. bieneusi* in the Qinghai-Tibetan Plateau area.

1. Introduction

Microsporidia comprises a highly diverse group of obligate intracellular parasitic fungi that infect an extremely wide range of vertebrates and invertebrates (Stentiford et al., 2016). To date, more than 220 genera and 1700 species of microsporidia have been described, among them, 17 species have been reported in humans (Li et al., 2019a; Shen et al., 2020). Of which, *Enterocytozoon bieneusi* is the most frequently introduced microsporidiosis agent that can cause diarrhea, wasting and/or malabsorption in animals and humans worldwide (Stark et al., 2009). The pathogen was first discovered in an AIDS patient suffering severe diarrhea in Haitian (Desportes et al., 1985). *E. bieneusi* has been detected in a wide range of hosts, including nearly all the mammalian and avian hosts, including humans, cattle, goats, sheep, horses, companion animals (cats and dogs), and so on (Lee et al., 2021; Zang et al., 2021; Zhang et al., 2018, 2019b, 2019c, 2020). The hosts are usually infected by the fecal-oral route and ingest contaminated food or water with spores of *E. bieneusi* (Lee et al., 2021).

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For detection of *E. bieneusi*, nested PCR for the ribosomal internal transcribed spacer (ITS) target gene has been widely used (Li et al., 2020; Santin and Fayer, 2011). Microscopy testing of *E. bieneusi* is challenging due to its extremely small spore size of approximately 1.0 μm, various shapes and in vitro culture method (Li and Xiao, 2021). By sequencing analysis of the ITS gene of *E. bieneusi*, more than 500 genotypes belonging to 11 genetic groups have been identified (Udonsom et al., 2019). Group 1 has been evaluated as major zoonotic group (Li et al., 2019a), which is the greatest group composed of more than 300 genotypes, containing most genotypes infecting humans and animals, such as genotypes D, EbpC, and Type IV (Li et al., 2020). While, group 2 has been regarded as the second largest cluster mainly found in ruminants, genotypes such as BEB6, BEB4, J and I, which have been mostly considered adapted to cattle, deer, sheep, goats, but also found in human (Li et al., 2019a). In addition, a small number of genotypes clustering in group 6 was found in human (Zhang et al., 2021). Generally, group 3 to group 11 contained fewer genotypes with host-specific characterization and posed a minor or unknown public health threat (Li and Xiao, 2021).

In the Qinghai-Tibetan Plateau area, Qinghai Lake is the largest saline lake in China. The lake's length is 105 km, its width is 63 km, and its height is 3196 m above sea level, which covers an area of 4583 km^2 surrounded by a 360 km coast line. Around the lake, there are many domesticated livestocks, Tibetan sheep and yaks, as well as amounts of wildlife, such as wild birds, pikas, marmots, blue sheep (*Pseudois nayaur*) and Przewalski's gazelle (*Procapra przewalskii*). Both blue sheep and Przewalski's gazelle are endemic species belonging to the Qinghai-Tibetan Plateau, and Przewalski's gazelle distribution points mainly surround Qinghai Lake (Gao et al., 2020; Shen et al., 2021). Every year, from April to June, many wild migratory birds fly back to the wetland of the Qinghai Lake. Through the Central Asian-Indian and East Asian-Australian flyways, Qinghai Lake serves as a breeding location for wild birds flying to Australia, India, and Southeast Asia (Jian et al., 2021).

To date, limited data about *E. bieneusi* infections in Tibetan sheep, yak, pika, wild Himalayan marmots, and Alashan ground squirrels had been reported (Liu et al., 2021; Xu et al., 2020; Zhang et al., 2018), but no information about infection with *E. bieneusi* from Przewalski's gazelle, blue sheep and wild birds has been reported. The study was conducted to detect *E. bieneusi* in domesticated livestock and wildlife around Qinghai Lake to enrich the epidemiological and genotyping data, evaluate the mutual transmission risk between domesticated livestock and wildlife, and assess the possibility of infection risk to humans.

2. Materials and methods

2.1. Collection of samples and DNA extraction

In this study, a total of 829 fresh fecal samples were collected around Qinghai Lake from April to September 2021, including wild birds (n = 227), wild blue sheep (n = 96), wild Przewalski's gazelle (n = 268), yaks (n = 128), and Tibetan sheep (n = 110). All the sampling sites were chosen to coexist with livestock and wildlife, and the animals probably shared water sources and/or grasslands.

Sampling sites were selected where domesticated animals and wild animals could share water sources and/or grasslands, mainly in southern Qinghai Lake (Gonghe County), northern Qinghai Lake (Gangcha County), and northeastern Qinghai Lake (Haiyan County). In these sites, there were many domesticated yaks and Tibetan sheep, as well as wetland environments, where a large number of migratory birds stayed for living and migrating, as well as mountain slopes and cliffs where blue sheep lived. Przewalski's gazelle was an endemic species of the Qinghai Lake region. Fresh fecal samples were collected into tubes containing 2.5% potassium dichromate, transferred to the laboratory in Xining, and stored at 4 °C. Samples of domesticated animals can be accurately identified. According to the fecal sample morphology, bird fecal samples were collected near water sources and bird gathering places. Similarly, samples from Przewalski's gazelle were also collected at the gathering place and blue sheep mainly appeared in some cliffs, so we followed them to collect samples.

After washing the fecal samples three times to remove potassium dichromate solution, genomic DNA from feces was extracted using the QIAamp Fast DNA Stool Mini Kit (QIAGEN, Germany) according to the manufacturer's manual, with the addition of 10 freeze-thaw cycles. The DNA samples were eluted with 80 μL of elution buffer and stored at −20 °C until used for PCR analysis.

2.2. Screening of E. bieneusi by nested-PCR

All DNA samples were screened by nested-PCR with the outer primers EBITS3/EBITS4 and inner primers EBITS1/EBITS2.4 for amplifying the 390 bp fragment of the ITS gene were conducted according to a previous study in 2002 (Buckholt et al., 2002). PCR primers and cycling protocols to amplify target sequences were described in Supplementary Table 1. The secondary PCR products were tested by 1.5% agarose gel containing 1 × GelStain (TransGen Biotech, China), and visualized using a G: BOX F3 Gel Documentation System (Syngene, UK).

2.3. Sequencing and phylogenetic analysis

The PCR products of positive samples were purified using the EasyPure Quick Gel Extraction Kit (TransGen Biotech, China), and cloned into *E. coli* DH5α using the pMD-19T Easy Vector System (Takara, Japan). Then positive clones were sent to SUZHOU GENEWIZ Company (Suzhou, China) for sequencing. The obtained sequences were confirmed by a BLASTn search in the GenBank, and then submitted to GenBank. The phylogenetic analyses were performed by the maximum likelihood statistical method and bootstrap analysis with 1000 replications using MEGA6 (Kimura 2-parameter model).

2.4. Statistical analysis

Statistical significance was defined as P values less than 0.05 ($P < 0.05$).

The 95% confidence intervals (CIs) werecalculated using the OpenEpi software program (http://www.openepi.com/Proportion/Proportion.htm).

3. Results

3.1. Prevalence of E. bieneusi

PCR detection based on the ITS gene revealed that the overall infection rate was 2.77% (23/829) for *E. bieneusi* (Table 1). Furthermore, from the five different hosts species, the Tibetan sheep with 9.09% (10/110) had the highest infection rate, followed by yaks with 3.91% (5/128), wild birds with 2.20% (5/227), wild blue sheep with 1.04% (1/96) and Przewalski's gazelle with 0.75% (2/268) (Table 1).

Table 1
Molecular detection of *E. bieneusi* in fecal samples from different animals.

Animals	No.Tested	No. Positive (%)	95%CI
Tibetan sheep	110	10 (9.09)	5.01–15.93
Yak	128	5 (3.91)	1.68–8.82
Bird	227	5 (2.20)	0.94–5.05
Blue sheep	96	1 (1.04)	0.18–5.67
Przewalski's Gazelle	268	2 (0.75)	0.20–2.68
Total	829	23 (2.77)	1.81–4.20

3.2. Identification of genotype

A total of twenty-three *E. bieneusi* ITS gene sequences were generated in this study. The obtained sequences were submitted to GenBank (accession numbers: OP723426-OP723448) (Table 2). For Tibetan sheep, nine different genotypes were identified out of ten isolates, including the known genotype BEB6, J, and seven novel genotypes (named CHS18-CHS24). Genotype BEB6 was the most common (20%, 2/10), followed by each positive sample of J, and CHS18-CHS24. The novel genotypes had one to four nucleotide differences from known genotypes (Table 3).

Among the five *E. bieneusi*-positive specimens from yaks, three genotypes were found, two known genotypes (BEB4 and J), and two novel genotypes (named CHN15 and CHN16). The occurrence frequency of genotype BEB4 (40%, 2/5) was the highest, followed by genotype J (20%, 1/5), the novel CHN15 (20%, 1/5) and CHN16 (20%, 1/5). The CHN15 genotype had one nucleotide mutation compared with genotype J, and the CHN16 genotype had three nucleotide mutations compared with genotype BEB6 (Table 3).

Of five positive samples from wild birds, five different genotypes were discovered: three known genotypes (EbpC, J and NCF2) and two novel genotypes (named CHWB1 and CHS24). The novel genotype CHS24 from bird shared the same nucleotide sequence with isolate CHS24 from Tibetan sheep. The genotype CHWB1 was similar to CHG7, with six nucleotide substitutions (Table 3).

From the only blue sheep-positive sample, one novel genotype (named as CHWBS1) was detected. The CHWBS1 genotype had twelve nucleotide differences from the COS-I genotype (Table 3). For two positive samples from Przewalski's gazelle, two genotypes were confirmed: one was known as genotype J, and the other was a novel genotype named CHWPG1, which had two nucleotide mutations compared with the genotype J (Table 3).

3.3. Phylogenetic analysis

From the phylogenetic analysis, five isolates belonged to zoonotic group 1, and the others were clustered into group 2. The five isolates belonging to group 1 included those from wild bird (n = 4) and Tibetan sheep (n = 1). The other eighteen samples clustered into group 2: wild bird (n = 1), wild blue sheep (n = 1), wild Przewalski's gazelle (n = 2), domesticated yak (n = 5) and Tibetan sheep (n = 9) (Fig. 1).

Table 2
Genotype of *E. bieneusi* in positive samples from different hosts.

GenBank Accession No.	Host	Genotype
OP723426	Tibetan sheep	BEB6
OP723427	Tibetan sheep	BEB6
OP723428	Tibetan sheep	J
OP723429	Tibetan sheep	**CHS18**
OP723430	Tibetan sheep	**CHS19**
OP723431	Tibetan sheep	**CHS20**
OP723432	Tibetan sheep	**CHS21**
OP723433	Tibetan sheep	**CHS22**
OP723434	Tibetan sheep	**CHS23**
OP723435	Tibetan sheep	**CHS24**
OP723436	Yak	BEB4
OP723437	Yak	BEB4
OP723438	Yak	J
OP723439	Yak	**CHN15**
OP723440	Yak	**CHN16**
OP723441	Bird	EbpC
OP723442	Bird	J
OP723443	Bird	NCF2
OP723444	Bird	**CHWB1**
OP723445	Bird	**CHS24**
OP723446	Przewalski's Gazelle	J
OP723447	Przewalski's Gazelle	**CHWPG1**
OP723448	Blue sheep	**CHWBS1**

Table 3
Variations in ITS region sequences of SSU rRNA genes of *E. bieneusi* isolates identified in this study and comparison with five known genotypes.

Genotype		Nucleotide at position																								GenBank Accession No.
		12	13	27	32	52	60	87	93	123	133	154	162	167	176	179	183	191	207	208	215	221	223	225	237	241	249	264	288	292	299	306	318	324	
known	BEB6	–	–	T	–	–	–	–	–	–	–	C	T	–	–	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	T	MK322762
novel	**CHS18**	–	–	C	–	–	–	–	–	–	–	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	OP723429
	CHN16	–	–	–	–	–	–	–	–	–	–	–	C	–	–	–	–	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C	OP723440
known	COS-I	–	A	–	–	–	A	–	A	–	T	–	–	–	T	G	T	–	G	T	–	A	T	G	G	G	–	–	–	T	–	G	G	–	MK322763
novel	**CHS19**	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	G	–	–	–	–	–	–	–	–	–	–	–	–	OP723430
	CHS20	–	–	–	–	–	–	–	–	–	–	–	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	OP723431
	CHS21	–	–	–	–	–	–	–	G	–	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C	–	–	–	–	OP723432
	CHWBS1	–	Del	–	–	–	C	–	–	–	–	–	–	–	–	A	A	–	C	A	–	–	C	C	C	C	–	–	–	–	–	T	T	–	OP723448
known	CHS3	–	–	–	T	T	–	–	–	–	–	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	MK573331
novel	**CHS22**	–	–	–	C	C	–	–	–	–	–	–	–	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	OP723433
	CHS23	–	–	–	–	–	–	–	–	–	–	–	–	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	OP723434
known	CHG7	A	–	–	–	–	–	T	–	A	–	–	–	–	–	–	–	–	–	–	T	–	–	–	–	–	G	T	–	–	–	–	–	–	KP262358
novel	**CHS24**	–	–	–	–	–	–	C	–	G	–	–	–	–	–	–	–	–	–	–	C	–	–	–	–	–	A	–	–	–	–	–	–	–	OP723435/OP723445
	CHWB1	T	–	–	–	–	–	C	–	G	–	–	–	–	–	–	–	–	–	–	C	–	–	–	–	–	A	C	–	–	–	–	–	–	OP723444
known	J	–	–	–	–	–	–	–	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	G	–	G	–	–	–	MT193868
novel	**CHN15**	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	OP723439
	CHWPG1	–	–	–	–	–	–	–	–	–	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C	–	–	–	OP723447

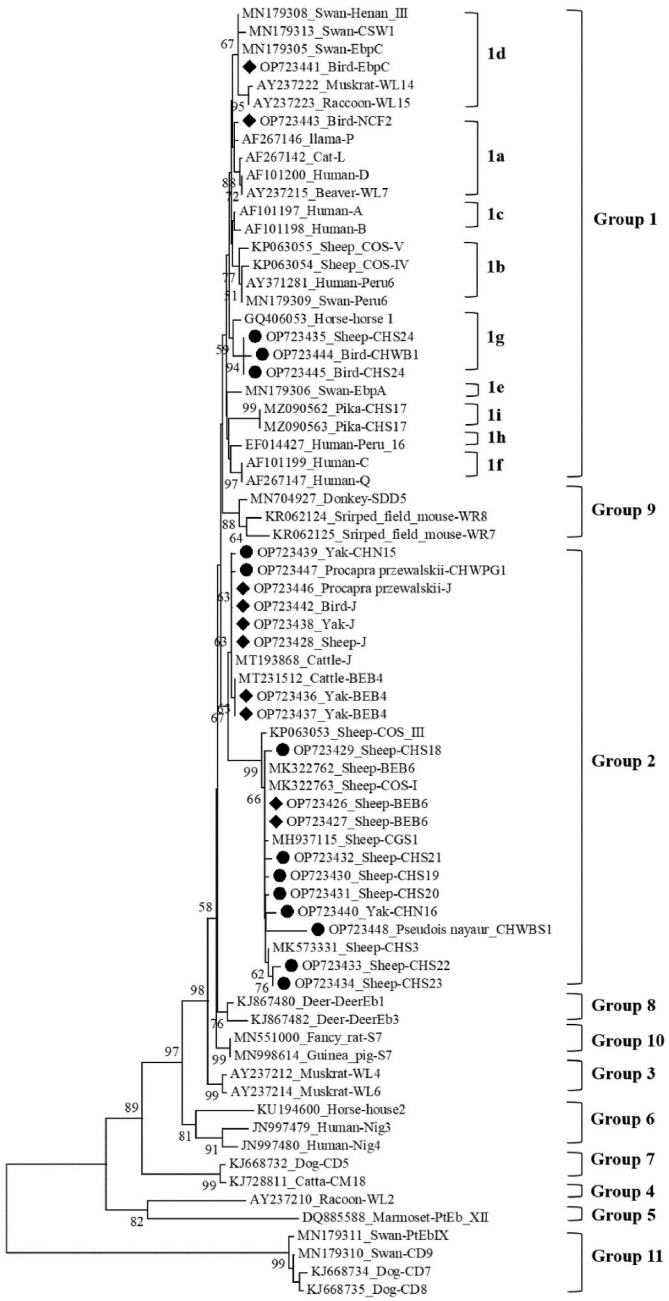

Fig. 1. Phylogenetic tree of *E. bieneusi* isolates and reference based on ITS region sequence of small-subunit ribosomal RNA (SSU rRNA) genes.

4. Discussion

In this study, the infection rate of *E. bieneusi* in Tibetan sheep was 9.09% (10/110), which was higher than another study from sheep in East-central China (3.4%, 28/832) (Li et al., 2019b), lower than that from natural grazing Tibetan sheep (15%, 93/620) in Tibet (Chang et al., 2020), and lower than that in another report from sheep in 11 provinces of China (20.4%, 194/953) (Yang et al., 2018). The prevalence in yaks (3.91%, 5/128) was higher than that in yellow cattle (2.5%, 11/442) and yaks (0, 0/76) in Tibetan (Wu et al., 2020), but lower than that in previous studies in Qinghai yaks (7%, 23/327 and 19.8%, 80/405) (Ma et al., 2015; Zhang et al., 2019a).

The infection in wild animals was relatively lower than that in livestock. In wild birds, 2.20% (5/227) showed a slightly lower infection rate than in captive birds (3.5%, 3/85) in Brazil (da Cunha et al., 2017) and a lower infection rate than in whooper swans (7.49%, 35/467) from the Swan Wetland Park in Henan Province (Wang et al., 2020). The difference in prevalence between captive and wild birds may be caused by different lifestyles and immunity.

To our knowledge, this is the first report about *E. bieneusi* infection in Przewalski's gazelle and blue sheep. It demonstrated that these animals were also suitable animal reservoirs in the epidemiology of *E. bieneusi*. The occurrence rates in wild blue sheep and Przewalski's gazelle were 1.04% (1/96) and 0.75% (2/268), respectively, which were lower than those in wild rodents (11.6%, 62/536) from six provinces in China (Ni et al., 2021), in wild rhesus macaques (11.7%, 38/324) in China (Yu et al., 2022), in wild boars (7.7%, 1/13), in sika deer (8.2%, 9/110), and in dogs (3.2%, 2/62) from Yichun, northeast China (Zhou et al., 2022). The low probability in wild Przewalski's gazelle and blue sheep was attributed to different geographical environments and a lower chance of contact with people and other domesticated animals.

For the phylogenetic analysis, four genotypes (EbpC, NCF2, CHWB1 and CHS24) from wild birds and one genotype (CHS24) from Tibetan sheep clustered to group 1, which means that wild birds and domesticated sheep can carry and have the potential to transmit the pathogens to each other, to other species and even to humans. The other samples all belonged to group 2, including genotypes from yaks (J, BEB4, CHN15, CHN16), blue sheep (CHWBS1), Przewalski's gazelle (J, CHWPG1), wild birds (J) and Tibetan sheep (J, BEB6, CHS18-CHS23). According to this study, genotype J was discovered in almost all the animals except blue sheep, so genotype J was the most widely distributed among different animal species. This pathogen can spread from one species to another, probably because of its geographical proximity. In addition, based on one previous review, genotypes EbpC and J identified in this study were also found in humans in China. In different areas, such as Harbin, Henan, Shanghai, there were detected genotype EbpC belonging to group 1 of *E. bieneusi* infecting human. Genotype J clustering in group 2 also were discovered in human in Changchun (Wang et al., 2018). But the data of *E. bieneusi* genotypes from human in Qinghai-Tibetan Plateau area has not been reported yet.

According to previous studies, the water sources can also be detected as positive for *E. bieneusi* (Hu et al., 2014; Yamashiro et al., 2017). In one study, 178 river water samples were collected from the upper Huangpu River, and a total of 56 (31.5%) samples were PCR-positive for *E. bieneusi* and genotype EbpC was the most common (Hu et al., 2014). Therefore, in our study, there was lack of water sources collected to detect *E. bieneusi*. In these sampling areas, there were also some other wild animals, such as pikas and marmots. In accordance with relevant research, 33 fresh fecal samples of plateau pikas were collected from the Qinghai Plateau area, and 5 (15.2%, 5/33) isolates were positive for *E. bieneusi* (Liu et al., 2021). For further study, we could collect more samples from more animal species to analyze the infection rate of *E. bieneusi* and its prevalent genotypes.

This study focuses on the prevalence of *E. bieneusi* in wild animals and livestocks in the same ecological environments around the Qinghai Lake regions, which provides decision-making basis and technical support for the prevention and control of microsporidosis caused by *E. bieneusi*, and further provides basic data for public health safety risk assessment in the Qinghai Plateau area.

5. Conclusions

This study provided unique data on the epidemiological reports and potential risk factors for *E. bieneusi* both in livestocks and wild animals around Qinghai Lake in the Qinghai-Tibetan Plateau area. Przewalski's gazelle and blue sheep were new and suitable hosts for *E. bieneusi*. Four genotypes EbpC, NCF2, CHWB1 and CHS24 from wild birds, and one genotype CHS24 from Tibetan sheep were clustered into group 1. The other genotypes J, BEB4, BEB6, CHN15, CHN16, CHS18-CHS23, CHWBS1 and CHWPG1 all belonged to group 2. The group 2 were mainly adapted to ruminants, so these genotypes were common found in

yaks, sheep, Przewalski's gazelle and blue sheep. One isolate genotype J from wild bird was also reminded that *E. bieneusi* could be distributed between these wild animals and livestocks due to living in the close geographical location. No other groups were discovered in this study. In our study, all the isolates were included in zoonotic group 1 and group 2, there was a high possibility of *E. bieneusi* transmitting to each other and even to humans. Further studies on more samples from animals, water sources and humans are also needed to better understand the molecular epidemiology and zoonotic potential of *E. bieneusi* in the Qinghai-Tibetan Plateau area.

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Author contributions

Y. Z. designed the study. Y.J. and X.Z. carried out the experiments and wrote the manuscript. G.W. contributed reagents and materials. Y. J., Geping W. and X.L. extracted DNA samples and made data curation. X.Z. and L.M. carried out the sequencing analysis and revised the manuscript. Y.J., Q. L. and C. L. coordinated work for samples collection. All authors have read and agreed to the published version of the manuscript.

Institutional review board statement

This study was carried out in accordance with the Law of the People's Republic of China on Wildlife Protection. The protocol of the current study was reviewed and approved by the Institutional Animal Care and Use Committee of the Qinghai Academy of Animal Sciences and Veterinary Medicine and conducted under permission of the Forestry and Grassland Bureau of Qinghai Province. No animals were harmed during the experimental process.

Informed consent statement

Not applicable.

Data availability statement

Not applicable.

Declaration of competing interest

The authors declare that they have no conflicts of interest with the contents of this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2023.04.012.

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