

Novel genotypes of *Cryptosporidium* and *Enterocytozoon bienersi* detected in plateau zokors (*Myospalax baileyi*) from the Tibetan Plateau

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ARTICLE INFO

Keywords:

Plateau zokor
Cryptosporidium sp.
Enterocytozoon bienersi
Novel genotypes
Host-adapted

ABSTRACT

The plateau zokor (*Myospalax baileyi*) is a small subterranean rodent endemic to China that lives alone in sealed underground burrows at altitudes ranging from 2000 to 4200 m above sea level on the Tibetan Plateau. Due to the unique environmental factors in the Tibetan Plateau, intestinal parasites in the local population may be more likely to develop host-adapted genotypes. We therefore conducted an epidemiological survey of common intestinal parasites in plateau zokors on the Tibetan plateau to estimate their actual gastrointestinal parasite status.

Two areas with high populations of plateau zokor in Xunhua County, Qinghai Province were selected as sampling sites, and a total of 98 zokors were trapped. Four parasites, *Cryptosporidium* spp., *Enterocytozoon bienersi*, *Giardia lamblia* and *Blastocystis hominis*, were tested in the faecal samples. The results showed that a new genotype of *Cryptosporidium* sp. was identified by amplification and sequencing of a portion of the small subunit ribosomal RNA (SSU rRNA) gene with an infection rate of 1.0% (1/98), and new genotypes of *E. bienersi* were identified by amplification and sequencing of a portion of the internal transcribed spacer (ITS) region of the ribosomal RNA gene sequences with an infection rate of 4.1% (4/98). Neither of the two intestinal parasites, *G. lamblia* and *B. hominis*, was detected.

1. Introduction

The plateau zokor (*Myospalax baileyi*) is a small subterranean rodent endemic to China that lives alone year-round in sealed burrows underground at altitudes of 2000–4200 m on the Tibetan Plateau (Kang et al., 2020a; Pu et al., 2019). Faced with a harsh environment of high humidity, limited oxygen, high CO₂ concentration, low temperature, and food scarcity, the body structure and physiological functions of Plateau zokor have evolved to adapt to the subterranean tunnel system (Shao et al., 2015). It is now become a good model for studying differentiation, selection, and species formation (Kang et al., 2022).

Cryptosporidium spp. and *E. bienersi* have been shown to have multiple host-adapted genotypes (Song et al., 2021; Xiao et al., 2002). *Cryptosporidium* sp. is a widespread zoonotic parasite, the main symptom of which is diarrhoea, capable of infecting 240 species of animals, including humans, and can cause serious public health emergencies in outbreaks (Ryan et al., 2018; Zahedi and Ryan, 2020). *Enterocytozoon*

bienersi are a group of intracellular, specific parasitic eukaryotes that can infect almost all vertebrates, including humans, are often associated with HIV or AIDS patients, making them an important opportunistic pathogen in humans (Li et al., 2019; Stentiford et al., 2016). *Giardia lamblia* is a genus of intestinal flagellates that infect a wide range of vertebrate hosts, and are found in a variety of mammals, including humans, pets and livestock (Feng and Xiao, 2011). *Blastocystis hominis* is a globally distributed intestinal protozoan that infects humans and many species of animals (Wang et al., 2013).

Compared to some terrestrial rodents, plateau zokors are less active, and their unique geographic location and living habits make them often an ideal model for studying phenotypic and ecological adaptations (Kang et al., 2020b). This also leads to a low probability of exogenous anthropogenic influences and pathogen invasion in plateau zokors, and intra-population intestinal parasites may be more likely to occur to produce host-adapted genotypes (Cao et al., 2014; Zhao et al., 2014). Therefore, we investigated the infection status of plateau zokors with

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<https://doi.org/10.1016/j.ijppaw.2022.11.002>

Received 12 August 2022; Received in revised form 9 November 2022; Accepted 9 November 2022

Available online 10 November 2022

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Cryptosporidium spp., *E. bienersi*, *G. lamblia* and *B. hominis* on the Tibetan plateau.

2. Materials and methods

Ethical statement

This study was conducted in accordance with the Guidelines for the Care and Use of Animals in Research published by the Institute of Zoology, Chinese Academy of Sciences. This study was reviewed and approved by the Animal Ethics Committee of the Institute of Zoology, Chinese Academy of Sciences (2019FY100300-03).

2.1. Sample capture of plateau zokor

The Qinghai subspecies of Plateau zokor (*Myospalax fontanierii baileyi*) was selected as the host for the study, and two regions with high populations of plateau zokor in Xunhua County, Qinghai Province were selected as the sampling sites. In September 2021, we went to each of these two sites and carried out rodent trapping using the rope trap method. A total of 30 zokors were captured at site 1 (35°38'46.3 "N 102°15'02.0 "E altitude: 3553.28m) and 68 zokors were captured at site 2 (3211.91m 35°41'48.7 "N 102°14'29.1 "E). Captured plateau zokors were euthanised with isoflurane and then taken back to the local laboratory for risk assessment. After confirming that there was no potential biosafety risk, the autopsy was performed, and gender and location were recorded, as shown in Table 1.

2.2. Microscopic examination

Parasite isolation and purification was an improvement on the method described by Zajac et al. (2021). In short, the collected feces were added to a sucrose solution and then floated and shaken and mixed, and then centrifuged to take the liquid film on the surface and place it for observation under an optical microscope.

2.3. Stool sample collection and DNA extraction

Rectal contents were collected into 2 mL sterilized centrifuge tubes, tubes were labeled, placed in liquid nitrogen for quick freezing, and then transported to the laboratory in Beijing using dry ice and stored in a −80 °C refrigerator. Total genomic DNA was extracted from 200 mg Stool according to the instruction of E.Z.N.A.® Stool DNA Kit Stool Whole Genome Extraction Kit (OMEGA Biotek INC4015-01, USA). The extracted Stool DNA was stored in a refrigerator at −20 °C for further PCR assays.

The small subunit ribosomal RNA (SSU) gene of *Cryptosporidium* spp. (Xiao et al., 2001), the internal transcribed spacer (ITS) of the ribosomal RNA gene of *E. bienersi* (Buckholt Michael et al., 2002), the β-giardin gene of *G. lamblia* (Lalle et al., 2005) and the SSU rRNA gene of *B. hominis* (Wang et al., 2013) were examined under nested PCR according to previous studies, respectively. Primer information and PCR procedures are detailed in Table 2. Positive control and negative control were added in each amplification. Amplified DNA was added to 1.5%

agarose containing Gel-Red for electrophoresis. Finally, the results of electrophoresis were observed and analyzed by gel imaging system and photographed.

2.4. Gene sequencing and phylogenetic analysis

The secondary PCR positive products were sequenced and spliced in both directions by Shenzhen Huada Gene Co Ltd (Beijing, China). After sequencing results were compared, DNAMAN software was used to correct splicing and assembly. We selected the host genotypes and *Cryptosporidium* sp. Environment sequences including chipmunk, muskrat, *Microtus arvalis*, muskrat genotype II, Brandt's vole genotype V, *C. hominis*, *C. ubiquitum* and others as reference genes. Due to the significant sequence differences among the copies of *Eimeria* SSU rRNA gene, the sequences of the three *Eimeria* species were selected for rooting of SSU rRNA phylogenetic trees. Similarly, samples including those covering Ebpc WbEb1 B13, B18, MUL5, D, CDZ211, JLNB-3, JLNB-6, horse 1, 5, ST32, Peru 16, BEB4, WbEb4, YZZ157, MS2, BEB6, BEB7, C4 and other taxa were selected as reference genes and *Enterocytozoon epatopenaei* was used as an outgroup control in the construction of the tree and was used for rooting of the ITS gene phylogenetic tree. *Cryptosporidium* and *Enterocytozoon* species/genotypes were determined by aligning with reference sequences available in GenBank database with the ClustalX 2.1 software package. The phylogenetic relationships of *Cryptosporidium* sp. and *E. bienersi* were constructed using the maximum-likelihood (ML) under MEGA 7.0 (Mello, 2018) to construct phylogenetic evolutionary trees, while the reliability was checked using Bootstrap with 500 replicates.

3. Results

3.1. *Cryptosporidium* spp., *E. bienersi*, *G. lamblia* and *B. hominis* infection rates in plateau zokors

The microscopic examination of the collected fecal samples showed that cysts and oocysts of *Cryptosporidium* spp., *E. bienersi*, *G. lamblia* and *B. hominis* were not detected. Then PCR was used to detect *Cryptosporidium* sp. SSU gene, *E. bienersi* ITS gene, *G. lamblia* β-giardin gene and *B. hominis* SSU rRNA gene by amplification in nested PCR, and only the samples after successful sequence alignment in the sequencing results were considered as positive samples. Of 98 samples, a total of 4 samples gave bands at the right place in the PCR for the *Cryptosporidium* spp. PCR, but only one could be successfully sequenced. And a total of 6 samples gave bands at the right place in the PCR for the *E. bienersi* PCR, 4 samples could be successfully sequenced. *Cryptosporidium* spp. was not detected at site 1 (0/30), 1.5% (1/68) at site 2 and 1.0% (1/98) overall. *E. bienersi* were detected at site 1 in 13.3% (4/30), not found positive at location 2 (0/68) and total infection was 4.1% (4/98).

The sequences of the 18S rRNA locus of *Cryptosporidium* sp. QH219-58 were sequenced to 853bp and submitted to GenBank to obtain the sequence accession number: **OM403638**. The ITS sequences of *E. bienersi* QH219-5, QH219-9, QH219-13, QH219-21 were 397bp, 393bp, 392bp and 392bp, respectively. The sequences were submitted to GenBank and the sequence accession numbers were obtained: **OM406189**, **OM406190**, **OM406191** and **OM406192**.

3.2. Phylogenetic analysis of SSU rRNA gene in *Cryptosporidium* sp

A phylogenetic tree was constructed by comparing with SSU rRNA gene sequence of reference *Cryptosporidium* sp. (Fig. 1). The genotypes of *Cryptosporidium* sp. found in plateau zokors were significantly different from other known host genotypes of *Cryptosporidium*, aggregating with the *Cryptosporidium chipmunk* genotype I.

Table 1

Sampling location and sample data information.

Factors	Category	Sampling Location 1	Sampling Location 2	Total
Location	longitude and latitude	35°38'46.3"N 102°15'02.0"E	35°41'48.7"N 102°14'29.1"E	
	Altitude (meter)	3553.28m	3211.91m	
Gender	Female	14	47	61
	Male	16	21	37
Total		30	68	98

Table 2Forward and reverse primers used for the detection of *Cryptosporidium* sp., *E. bienersi*, *G. lamblia* and *B. hominis*.

Gene	Primers			Amplification protocol	Size	Ref
<i>Cryptosporidium</i> sp. SSU gene	1st	Forward	5'-TTCTAGAGCTAATACATGCG-3'	94 °C: 3 min, 35 cycles: (94 °C: 45 s, 55 °C: 45 s, 72 °C: 1 min) 72 °C: 7min	1325 bp	Xiao et al., 2001
	PCR	Reverse	5'-CCCATTTCCTTCGAAACAGGA-3'			
	2nd	Forward	5'-GGAAGGGTGTATTATTAGATAAAG-3'	94 °C: 3 min, 35 cycles: (94 °C: 45 s, 55 °C: 45 s, 72 °C: 1 min) 72 °C: 7min	864 bp	
	PCR	Reverse	5'-AAGGAGTAAGGAACAACCTCCA-3'			
<i>E. bienersi</i> ITS gene	1st	Forward	5'-GGTCATAGGGATGAAGAG-3'	94 °C: 5 min, 35 cycles: (94 °C: 30 s, 57 °C: 30 s, 72 °C: 40 s) 72 °C: 7min		Buckholt et al., 2002
	PCR	Reverse	5'-TTCGAGTTCTTTCGCGCTC-3'		435 bp	
	2nd	Forward	5'-GCTCTGAATATCTATGGCT-3'	94 °C: 5 min, 35 cycles: (94 °C: 30 s, 55 °C: 30 s, 72 °C: 40 s) 72 °C: 7min		
	PCR	Reverse	5'-ATCGCCGACGGATCCAAGTG-3'		390 bp	
<i>G. lamblia</i> β-giardin gene	1st	Forward	5'-AAGCCCGACGACCTACCCGAGTGC-3'	94 °C: 5 min, 35 cycles: (94 °C: 30 s, 65 °C: 30 s, 72 °C: 40 s) 72 °C: 7min	753 bp	Lalle et al., 2005
	PCR	Reverse	5'-GAGGCCGCGCTGGATCTTCGAGACGAC-3'			
	2nd	Forward	5'-GAACGAACGAGATCGAGGTCCG-3'	94 °C: 5 min, 35 cycles: (94 °C: 30 s, 55 °C: 30 s, 72 °C: 40 s) 72 °C: 7min	511 bp	
	PCR	Reverse	5'-CTCGACGAGCTTCGTGTT-3'			
<i>B. hominis</i> SSU rRNA gene	1st	Forward	5'-GGGATCCTGATCCTCCGAGGTTACCTAC-3'	94 °C: 5 min, 35 cycles: (94 °C: 30 s, 65 °C: 45 s, 72 °C: 40 s) 72 °C: 7min	1800 bp	Wang et al., 2013
	PCR	Reverse	5'-GGAAGCTTATCTGTTGATCCTGCCAGTA-3'			
	2nd	Forward	5'-GGAGGTAGTGGACAATAAATC-3'	94 °C: 5 min, 35 cycles: (94 °C: 30 s, 54 °C: 45 s, 72 °C: 40 s) 72 °C: 7min	1100 bp	
	PCR	Reverse	5'-CGTTCATGATGAACAATTAC-3'			

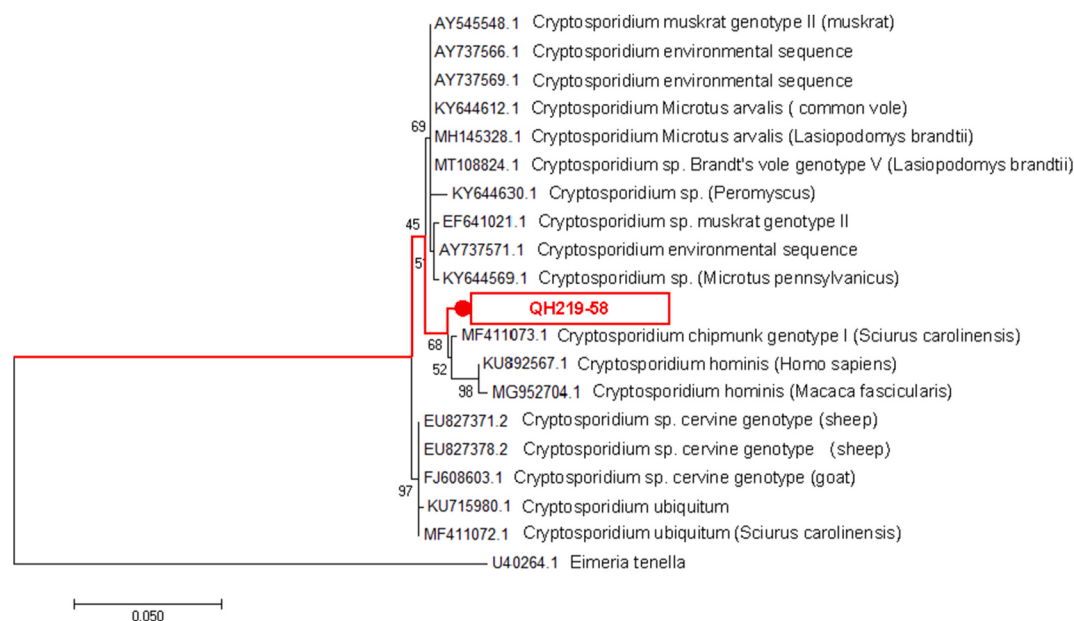


Fig. 1. Phylogenetic relationships of *Cryptosporidium* sp. genotypes identified in the present study and other known genotypes and species on GenBank was inferred by a maximum-likelihood phylogenetic analysis of SSU rRNA gene sequences using the Tamura 3-parameter model and with 500 replicates. The *Eimeria* (GenBank: U40264.1) were used as the outgroup. The red circles and squares indicate the novel genotypes identified in this study. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3. Phylogenetic analysis of ITS gene in *E. bienersi*

A phylogenetic tree was constructed by comparing and analyzing the ITS gene sequence with that of the reference *E. bienersi* (Fig. 2). The genotypes of *E. bienersi* detected in plateau zokor were significantly different from those of other known *E. bienersi* genotypes, forming a separate group.

4. Discussion

Rodents are considered to be important zoonotic hosts for *Cryptosporidium* spp. (Zhang et al., 2022) and *E. bienersi* (Ni et al., 2021). To

our knowledge, this study is the first time to investigate the infection status of *Cryptosporidium* sp. and *E. bienersi* in plateau zokors. Many studies have shown that *Cryptosporidium* sp. can infect humans and animals, and many *Cryptosporidium* sp. species/genotypes of public health significance have been identified. However, it has been rarely reported in rodents, especially in plateau zokors. These results indicate that the prevalence of *Cryptosporidium* sp. infection in plateau zokors was not only lower than that of the Chinese Qinghai vole (8.9%, 8/90) and plateau pika (6.25% (4/64) in the same region (Zhang et al., 2018), but also lower than that of rodent populations in other countries (Horčíková et al., 2019; Stenger et al., 2018). The global prevalence of *Cryptosporidium* sp. in rodents is estimated to be about 17% (Taghipour



Fig. 2. Phylogenetic relationships of *E. bieneusi* genotypes identified in the present study and other known genotypes deposited on GenBank was inferred by a maximum-likelihood phylogenetic analysis of ITS sequences using the Tamura 3-parameter model with 500 replicates. The *Enterocytozoon hepatopenaei* (GenBank: KR021167.1) was used as the outgroup. The blue triangle and squares indicate the novel genotypes identified in this study, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

et al., 2020), which is higher than our detection results. The *E. bieneusi* in plateau zokors is lower than the average infection rate of 10.0% (50/498) in rodents from five regions within Gansu Province (Xu et al., 2020).

In this study, neither *G. lamblia* nor *B. hominis* were detected positively, which may be related to the low number of parasite infections and the low intensity of infection. Based on these results we speculate that this may be related to the unique living environment and habits of plateau zokors. An increase in rodent population density can cause an increase in infectious disease infection rates (Krawczyk et al., 2020), but plateau zokors often live alone underground, reducing the probability of suffering from disease infestation. Coupled with the fact that intra-population encounters are mostly possible during spring breeding, low-frequency contact within populations reduces parasite transmission (Cai et al., 2018; Jonsson et al., 2010).

Most of the morphological differences between *Cryptosporidium* spp. and *E. bieneusi* species cannot be distinguished by the naked eye, and identification based on oocyst morphology alone has limitations, which can be compensated by molecular identification methods that can analyze genetic differences between different *Cryptosporidium* species at the genetic level (Ryan et al., 2014; Xiao, 2010). The 18S rRNA gene of *Cryptosporidium* sp. is responsible for encoding the multicopy repeat sequence of the SSU rRNA, which is approximately 1850 bp in length and is the marker gene with both informative and functional roles, making it the most widely used genotyping tool for identifying host-adapted *Cryptosporidium* (Xiao and Feng, 2017). Host adaptation is a general phenomenon in the genus *Cryptosporidium*, and specific

genotypes are usually associated with specific animal groups (Xiao et al., 2002). The new genotype identified in this study is evolutionarily related to *C. chipmunk* genotype I and *Cryptosporidium hominis*. *C. chipmunk* genotype I, originally identified in rodents (chipmunks, squirrels, and deer mice) and watershed runoff in New York, has been identified as a novel zoonotic pathogen in humans (Guo et al., 2015; Xu et al., 2019), and the disease has had small outbreaks in Sweden and poses a significant public health threat (Bujila et al., 2021). Therefore, the *Cryptosporidium* sp. found in plateau zokors may not only be a new host-adapted genotype, but also a novel zoonotic pathogen.

Sequence analysis of the ITS of the ribosomal RNA gene of *E. bieneusi* has been widely used in *E. bieneusi* typing studies (Widmer and Akiyoshi, 2010). Based on the high diversity of ITS, more than 500 genotypes have been identified, some of which are host-adapted genotypes (Karim et al., 2014, 2015; Santín and Fayer, 2011; Wei et al., 2019). It has been demonstrated that environmental and host isolation characteristics make differences in the distribution of *E. bieneusi* genotypes (Wei et al., 2019). New genotypes may exist in different hosts, for example, ten novel genotypes (WR1-WR10) were identified in wild rodents in Poland in 2015 (Perec-Matysiak et al., 2015). A study in the USA found that the *E. bieneusi* prevalent in animals from Texas in 2011 may be a prairie dog-specific genotype (Roellig et al., 2015).

In the present experiment, phylogenetic analysis using the ML method showed that all ITS representative gene sequences of *E. bieneusi* identified in this study were evolutionarily distant from the pre-existing nucleotide sequences, forming a separate branch, indicating that it may be a plateau zokor-specific genotype, and based on the differences in its

molecular characteristics and host specificity, we therefore named the newly identified genotype as zokor genotype of *Enterocytozoon bienersi*.

5. Conclusions

In summary, this study enriched the host range and genotype database of *Cryptosporidium* spp. and *E. bienersi* by molecular biological detection and phylogenetic analysis of host-adapted genotypes in plateau zokor populations in the Tibetan Plateau region, providing a new case for environmental isolation that may lead to special species reservoirs of more host-adapted genotypes of parasites.

Author contributions statement

Hongxuan He, Bin Hu contributed to the conception of the study; Jiamin Wang, Shuairan Zhang performed the experiment; Yanan Xing, Shuyi Han contributed significantly to analysis and manuscript preparation; Bo wang helped perform the analysis with constructive discussions; Bin Hu performed the data analyses and wrote the manuscript.

Data availability statement

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Declaration of competing interest

All the authors have no conflict of interest to declare.

Acknowledgments

This study was financially supported by the Strategic Priority Research Program of the Chinese Academy of Sciences (XDA19050204), National Key R&D Program of China, National Forestry and Grassland Administration, China, the Major Program of National Natural Science Foundation of China (No. 32090023), Beijing Innovation Consortium of Agriculture Research System (BAIC04-2021).

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